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(71)Applicant: MAX PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENCHAFTEN e.V. [DE/DE]; Bunsenstrasse 10, D-3400 Göttingen (DE).

(72) Inventors: LINDHOLM, Dan, Bjarne ; Otto-Dischner-Weg 13, D-8000 München 60 (DE). THOENEN, Hans, Friedrich, Erwin ; Kraeplin 4A, D-8000 München 40 (DE). HENGERER, Bastian ; Am Wasserbogen 39, D-8032 Grafelfing (DE).

(74) Agents: RUFF, M. et al.; Neckarstraße 50, D-7000 Stuttgart 1 (DE).

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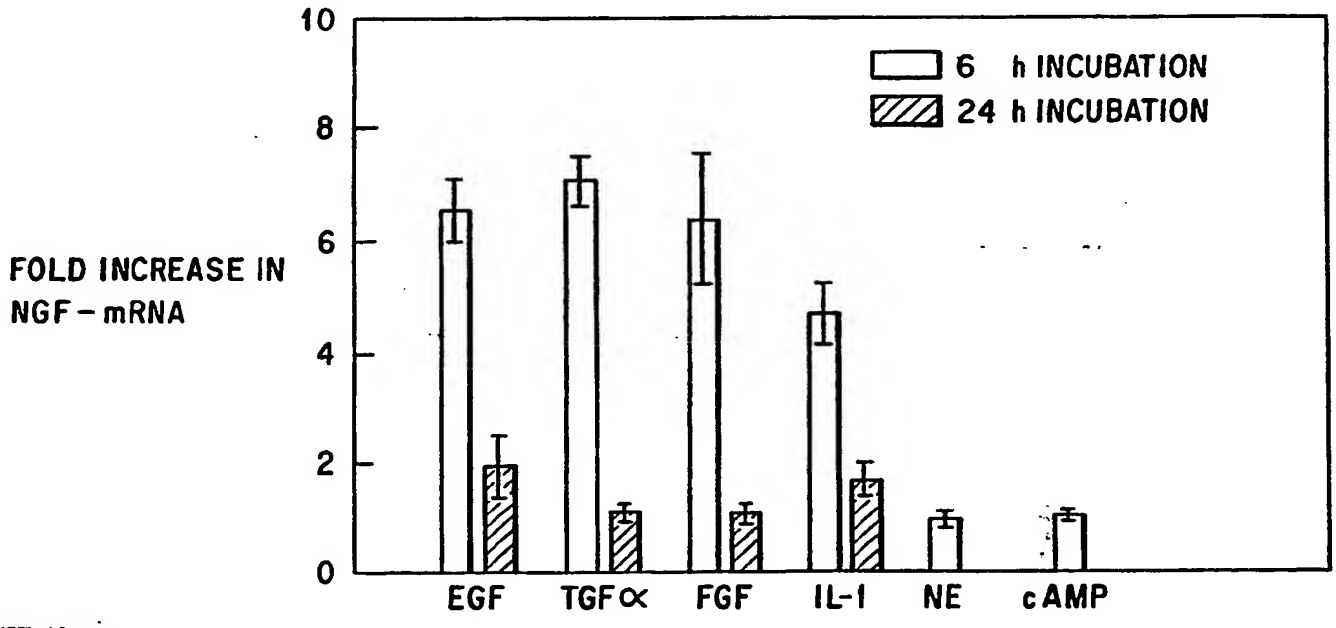

(57) Abstract

The present invention relates to a method for regulating levels of nerve growth factor in the central nervous system, and is based on the discovery that *in vivo* synthesis of nerve growth factor may be regulated by various cytokines. In specific embodiments, intracerebroventricular injection of interleukin 1, fibroblast growth factor, or $TGF\beta_1$ incr as synthesis of nerve growth factor and its mRNA. The methods of the present invention may be useful in the treatment of various neurologic diseases or disorders, including Alzheimer's disease.

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REGULATION OF NERVE GROWTH FACTOR SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM

1. INTRODUCTION

The present invention relates to a method for regulating levels of nerve growth factor in the central nervous system, and is based on the discovery that in vivo synthesis of nerve growth factor may be regulated by various cytokines. In specific embodiments, intracerebroventricular injection of interleukin 1, transforming growth factor-beta and/or fibroblast growth factor increase synthesis of nerve growth factor and its mRNA. The methods of the present invention may be useful in the treatment of various neurologic diseases or disorders, such as Altzheimer's disease.

2. BACKGROUND OF THE INVENTION

2.1. NERVE GROWTH FACTOR

Nerve growth factor (NGF) was discovered in the early 1950s. Extending earlier observations (Bueker, 1948, Anat. Rec. 102:369-389) that neurites grew out of dorsal root ganglia of a chick embryo into a mouse sarcoma implanted in the chick body wall, Levi-Montalcini and Hamburger (1951, J. Exptl. Zool. 116:321-362; 1953, J. Exptl. Zool. 123:233-278) showed that sympathetic ganglia also participated in the nerve ingrowth to the tumor, and demonstrated that the effects were due to a specific growth factor produced by the sarcoma.

The biochemistry, synthesis, and mechanism of action of NGF are reviewed by Greene and Shooter (1980, Ann. Rev. Neurosci. 3:353-402) and Thoenen and Barde (1980, Physiological Reviews 60:1284-1335). The NGF molecule consists of two noncovalently linked, identical peptide chains (Greene et al., 1971, Neurobiology 1:37-48; Angeletti et al., 1971, Biochemistry 10:463-469; Pignatti et al., 1975, J. Neurochem. 25:155-159). Each chain

contains 118 amino acid residues whose sequence has been determined (Angeletti and Bradshaw, 1971, Proc. Natl. Acad. Sci. U.S.A. 68:2417-2420; Angeletti et al., 1973, Biochemistry 12:100-115). The chains have NH_2-terminal serine residues, COOH-terminal arginine residues, three internal disulfide bridges, and two and three residues, respectively, of tyrosine and tryptophan. The amino acid sequence of the NGF chain is homologous to that of proinsulin in sections of the chain that correspond to the presumptive B and A chains of insulin (Frazier et al., Science 176:482-488). Moreover, one of the interchain disulfide bridges of insulin is conserved in the NGF structure. This relatedness extends also to the hormone relaxin (Scwabe and McDonald, 1977, Science 197:914-915) and to the insulin-like growth factors I and II (Rinderknecht and Humbel, 1978, J. Biol. Chem. 253:2769-2776).

The gene coding for NGF has been cloned, and its structural organization elucidated (Francke et al., 1983, Science 222:1248-1250; Scott et al., 1983, Nature 302:538-540; Ullrich et al., 1983, Nature 303:821-825).

2.1.1. NERVE GROWTH FACTOR IN THE PERIPHERAL NERVOUS SYSTEM

NGF is required for the normal development and maintenance of the sympathetic nervous system and certain sensory neurons (Levi-Montalcini, 1964, Science 143:105-110). The source of NGF for these neurons is their peripheral target tissues (Korsching and Thoenen, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3513-3516); NGF mRNA has been detected in various sympathetically innervated end organs (Sheldon and Reichardt, 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7951-7955; Heumann et al., 1984, EMBO J.

3:3183-3189), such as the adult male mouse submaxillary gland.

NGF acts as a retrograde messenger between effector and innervating neurons. Neuronal responses to NGF include increased neurite outgrowth and enlargement in cell body size (Levi-Montalcini, 1966, supra). In sympathetic ganglia, NGF brings about an increase in the synthesis of the enzymes tyrosine hydroxylase and dopamine β-hydroxylase (Thoenen et al. 1971, Proc. Natl. Acad. Sci. U.S.A. 68:1598-1602; Thoenen and Barde, 1980, Physiol. Rev. 60:1284-1335). Besides being a neurotropic factor for catecholaminergic peripheral sensory neurons, NGF also affects peptidergic neurons of the peripheral sensory system (Otten, 1984, Trends Pharmacol. 7:307-310; Yip and Johnson, 1984, Proc. Natl. Acad. Sci. 81:6245-1249).

Both in vitro and in vivo experiments have established that NGF plays a necessary role in the survival of sympathetic and some embryonic sensory neurons (Levi-Montalcini and Booker, 1960, Proc. Natl. Acad. Sci. U.S.A. 46:384-391). Pharmacological administration of NGF prevents neuronal death in the dorsal root ganglia neurons of newborn rats following sciatic nerve injury (Yip et al., 1984, J. Neurosci. 4:2986-2992).

2.1.2. NERVE GROWTH FACTOR IN THE CENTRAL NERVOUS SYSTEM

In contrast to the peripheral nervous system, the catecholaminergic neurons of the central nervous system (CNS) have been found to neither exhibit specific retrograde axonal transport of NGF (Schwab et al., 1979, Brain Res. 168:473-483; S[illegible]ilei and Schwab, 1984, Brain Res. 300:33-39) nor respond to NGF with the induction of tyrosine hydroxylase (Schwab et al., 1979, supra). Early evidence of NGF-responsive neurons in the CNS was

presented by Schwab et al. (1979 and 1984, supra), who injected radiolabelled NGF into the hippocampus and cortex of adult rats, and followed its subsequent distribution autoradiographically; NGF appeared to be transported specifically to the cell bodies of magnocellular cholinergic neurons in the basal forebrain. Iodinated NGF and anti-NGF receptor antibodies have been found to consistently bind to magnocellular cholinergic neurons (Richardson et al., 1986, J. Neurosci. 6:2312-3221; Taniuchi et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:1950-1954). Furthermore, repeated intraventricular injections of NGF in neonatal rats led to an increase in choline acetyltransferase (CAT) in the hippocampus, cortex, and septal area (Gnahn et al., 1983, Dev. Brain Res. 9:45-52). Brain regions not related to the magnocellular cholinergic system have been found to contain considerably lower levels of NGF (Korsching et al., 1985, EMBO J. 4:1389-1393).

The magnocellular cholinergic neurons of the basal forebrain project topologically to the neocortex, hippocampus, and olfactory bulb (Wainer et al., 1984, Neurochem. Int. 6:163-182). The learning ability of rats in spatial memory tasks is severely reduced by lesions of the cholinergic septohippocampal pathway, and can be restored by implants of fetal septal neurons into the hippocampus (Dunnet et al., 1982, Brain Res. 251:335-348). In Alzheimer's disease, cholinergic neurons of the basal forebrain nuclei are consistently affected in an early stage of the disease, and it appears that the cognitive deficits are predominantly determined by the impaired function of these cholinergic neurons (Hefti and Weiner, 1986, Ann. Neurol. 20:275-281). These clinical observations are in agreement with the observations of Bjorklund and collaborators (Fischer et al., 1987, Nature

329:65-68), that in aged rats there is a correlation between impaired cognitive functions and the extent of regressive changes in the cholinergic neurons of the basal forebrain nuclei.

Hefti and Weiner (1986, Ann. Neurol. 20:275-281) observed that degenerative changes of basal forebrain cholinergic nuclei after fimbria-fornix lesion could be prevented by NGF. In addition, Kromer et al. (1987, Science 233:214-216) showed that continuous infusion of NGF into the lateral ventricles of adult rats prevented neuronal death following bilateral injury of cholinergic neurons projecting from the medial septum to the dorsal hippocampus.

As in the periphery, the density of innervation by NGF responsive neurons is correlated with the levels of NGF and its mRNA (Korsching et al., 1985, EMBO J. 4:1389-1399; Whittemore et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:817-821; Large et al., 1986, Science 234:352-355). In contrast to the periphery, where a great variety of cells such as epithelial cells, smooth muscle cells, fibroblasts, and Schwann cells contribute to the synthesis of NGF in the projection fields of the NGF-responsive neurons (Bandtlow et al., 1987, EMBO J. 6:891-899), in the central nervous system, NGF synthesis has unambiguously been demonstrated for astrocytes only (Lindsay, 1979, Nature 282:80-82; Furukawa et al., 1986, Biochem. Biophys. Res. Commum. 136:57-63, 1987, Biochem. Biophys. Res. Commun. 142:395-402). Recently, in situ hybridization experiments have localized NGF-mRNA in cells of the rat hippocampus and neocortex (Rennert and Heinrich, 1986, Biochem. Biophys. Res. Commun. 138:813-818; Ayer-LeLievre et al., 1988, Science 240:1339-1341; Whittemore et al., 1988, J. Neurosci. Res. 20:403-410), opening up the possibility that in the central n rvous system (CNS)

neurons might contribute to NGF synthesis; however, the relative contribution of glial cells and neurons remains to be established.

3. SUMMARY OF THE INVENTION

The present invention is directed toward a method for regulating nerve growth factor levels in the central nervous system comprising the administration of cytokine(s), and the uses of such methods in the therapeutic treatment of various neurologic diseases and disorders.

The invention relates to the discovery that various cytokines, administered in vivo, are capable of altering the level of expression of NGF.

In particular embodiments, introduction of IL-1, TGF-β, TGF-α, or FGF into the CNS can increase the synthesis of NGF-mRNA and the production of NGF, and may be used in the treatment of neurologic diseases including but not limited to dementias, such as Alzheimer's disease.

The invention also relates to methods for regulating the levels of nerve growth factor in the central nervous system of a subject in which a substance is administered which alters the level of a cytokine that in turn alters the level of nerve growth factor. For example, glucocorticoids may be used to decrease levels of NGF in the central nervous system by inhibiting interleukin-1.

The present invention provides methods for the selection of cytokines that may be used to regulate NGF synthesis in effective therapeutic regimens.

3.1. LIST OF ABBREVIATIONS

bFGF: basic fibroblast growth factor
BSA: bovine serum albumin
DMEM: Dulbeccos Modified Eagles Medium
EGF: epidermal growth factor
FCS: fetal calf serum
GFAP: glial fibrillary acidic protein
IL-1: Interleukin 1
mRNA: messenger ribonucleic acid
NGF: nerve growth factor
PDGF: platelet derived growth factor
PBS: phosphate buffered saline
TGF-α: transforming growth factor-alpha
TGF-β: transforming growth factor-beta

4. DESCRIPTION OF THE FIGURES

FIGURE 1. Immunocytochemical characterization of purified astrocyte cultures using mouse monoclonal antibodies against and vimentin (a) and GFAP (c), (b) and (d) are unstained.

FIGURE 2. Immunocytochemical characterization of non-astrocyte cells in purified astrocyte cultures (a) using mouse monoclonal antibodies 04 and anti-Thy 1.1 and (c) testing for unspecific esterase reaction according to Koski et al. (1976, in "In Vitro Methods in Cell Mediated and Tumor Immunity" Academic Press, New York, p. 359); (b) and (d) unstained).

FIGURE 3. In situ hybridization of NGF-mRNA in mixed rat brain primary cultures (a) stained cells (b) autoradiograph showing in situ hybridization.

FIGURE 4. Effect of fetal calf serum on NGF and NGF-mRNA levels in purified astrocyte cultures, as determined by ELISA and quantitative Northern blot assay, respectively.

FIGURE 5. Results of quantitative Northern blot analysis showing the effect of EGF, TGF-α, FGF, IL-1β, norepinephrine (NE) and 8-bromo-cAMP on NGF-mRNA levels in purified astrocyte cultures.

FIGURE 6. NGF-mRNA levels in astrocyte cultures incubated with IL-1β over a 24-hour period, determined by quantitative Northern blot analysis.

FIGURE 7. Effect of TGF-β1, at a concentration of 5 ng/ml, on NGF mRNA levels in astrocyte cultures over a 48 hour period, as measured by Northern blot analysis.

FIGURE 8. Effect of various concentrations of TGF-β1 on NGF mRNA levels in astrocyte cultures, as measured by Northern blots.

FIGURE 9. Levels of NGF in astrocyte cultures incubated with IL-1β and various growth factors for 24 hours, measured by ELISA.

FIGURE 10. Levels of NGF-mRNA in rat hippocampus eight hours after intracerebroventricular injection of TGFα, FGF, TGF-β or IL-1β, as determined by quantitative Northern blot analysis.

FIGURE 11. Changes in NGF-mRNA levels in sciatic nerve after lesion and in nerve segments kept in culture; effect of dexamethasone.

Rat sciatic nerves were unilaterally transected and nerve segments collected after 6 hours (see Materials and Methods). Dexamethasone was injected intraperitoneally to some rats 2 hours before nerve lesion (hatched bars). Sciatic nerve segments were also cultured for 6 hours in the absence (open bars) or presence (hatched bars) of dexamethasone (1μM). Following incubation, the amount of NGF-mRNA in samples was determined by Northern blot analysis and by a scanning of the autoradiograms. Values are the

mean SD of more than three experiments. The bars denote the following groups of animals:
C: control, untreated rats;
D: dexamethasone-treated rats;
L: nerve lesion;
L+D: nerve lesion after dexamethasone;
S: nerve segments incubated in the presence of serum;
S+D: nerve segments cultured with serum and dexamethasone;
M: nerves cultured in medium (DMEM) only, without serum;
M+D: nerve segments cultured in DMEM in the presence of dexamethasone.

FIGURE 12. Effect of dexamethasone on NGF-mRNA levels in cultured fibroblasts.

Rat sciatic fibroblasts were treated with IL-1 (30 units/ml) or fetal calf serum (10%, final concentration) in the absence or presence of dexamethasone (1 μM). Following incubation for 3 hours (IL-1) or 4 hours (serum) total cellular RNA was prepared and subjected to Northern blot analysis. The autoradiogram shows the presence of NGF-mRNA (1.3 kb) and a shorter NGF transcript (0.51 kb) which was used as a standard to assess recovery (see Materials and Methods).
Lane 1: control, untreated cells;
lane 2: cells treated with dexamethasone;
lane 3: IL-1-treated cells;
lane 4: IL-1 and dexamethasone added together;
lanes 5-6: serum-treated cells;
lane 7: serum and dexamethasone together.

FIGURE 13. Time-course for the effect of dexamethasone on NGF-mRNA levels in fibroblasts.

Rat sciatic fibroblasts were kept in low serum and 10% fetal calf serum was added to induce NGF-mRNA. Dexamethasone (1 μM) was also added to the cultures either together with serum or at different times after serum addition. Following a total incubation time of 4 hours, cellular RNA was extracted and analyzed by Northern blots. The autoradiogram shows the presence of NGA-mRNA and the lower NGF transcript used as standard (see Materials and Methods)

Lane 1: Controls;
lane 2: serum alone for 4 hours;
lane 3: serum and dexamethasone added at the same time;
lane 4: dexamethasone added 1 hour after serum;
lane 5: dexamethasone added 2 hours after serum;
lane 6: dexamethasone added 3 hours after serum;
lane 7: low concentration of dexamethasone (0.01 μM) added together with serum.

FIGURE 14. Effect of dexamethasone on NGF promoter activity in transfected sciatic fibroblasts.

Rat sciatic fibroblasts were transfected overnight with the plasmid pBLPNCAT3 (see Materials and Methods). The cells were washed three times with PBS and incubated for 24 hours with 10% fetal calf serum in the presence or absence of dexamethasone (1 μM).

FIGURE 14 a. shows an autoradiogram of a typical experiment using serum and dexamethasone.

FIGURE 14 b. summarizes the results of three independent experiments.

M: denotes fibroblasts cultured in medium (DMEM) only.
S: are fibroblasts incubated with serum.
S+D: fibroblasts cultured with serum and dexamethasone.

M+D: are fibroblasts incubated in DMEM in the presence of dexamethasone.

FIGURE 15: Effect of TGF-β1 on NGF-mRNA in astrocytes.

Rat astrocytes were treated for 4 hours with TGF-β1 (2ng/ml) or with TGF-β1 and other agents. Cellular RNA was extracted and analyzed by Northern blots. The autoradiogram shows the presence of NGF-mRNA (1.3kb) and a shortened NGF transcript (0.51kb) added to the samples prior to RNA extraction.

Lane 1: Controls
Lane 2: TGF-β1
Lane 3: TGF-β1 in the presence of anti-TGF-β-antibodies
Lane 4: Cycloheximide (10 φg/ml)
Lane 5: TGF-β1 and cycloheximide (10 φg/ml)
Lane 6: TGF-β1 with actinomycine-D (10 φg/ml)

FIGURE 16: Time-course and dose-response relationship of NGF-mRNA induction in astrocytes by TGF-β1.

FIGURE 16a: Time-course NGF induction.

Astrocytes were treated with TGF-β1 (5ng/ml) for various periods of time. Values are means ±SD of three experiments and are expressed as pg NGF-mRNA/10^6 cells. The open circle represents control cells incubated for 48 hours without TGF-β1.

FIGURE 16b: Dose-response curve.

The incubation was carried out for 24 hours in the presence of various concentrations of TGF-β1. Values given represent the average of two determinations.

FIGURE 17: Effect of TGF-β1 on NGF promoter activity in transfected rat astrocytes.

Rat astrocytes were transfected with the plasmid pBLCAT3-NGF which contains the promoter region of mouse NGF. After washing, the cells were incubated

for 24 hours in the absence or presence of TGF-β1 and CAT activity was subsequently determined.

C: Controls

B1: Astrocytes incubated with TGF-β1.

FIGURE 18: Effect of TGF-β1 on nuclear labelling in astrocytes.

Isolated nuclei from control and TGF-β1 treated cells were incubated with radioactive UTP to label RNA, which was subsequently hybridized to cRNA probes spotted onto nylon filters. tRNA was used as an internal control.

B1: TGF-β1 transcript

NGF(+): Anti-sense NGF

NGF(-): Sense NGF transcript.

FIGURE 19: Presence and stimulation of TGF-β-mRNA in rat astrocytes.

Astrocytes were treated with TGF-β1 for 4 hours and cellular RNA was analyzed by Northern blots using a cRNA probe specific for TGF-β1. Astrocytes contain two transcripts of TGF-β1 (2.5kb and 1.9kb). MØ denotes 5 φg RNA isolated from rat macrophages known to express TGF-β1-mRNA.

FIGURE 19a: Effect of TGF-β1 on TGF-β1 gene transcription.

Astrocytes were transfected with the CAT expression vector containing the mouse TGF-β1 promoter region. The cells were incubated and analyzed as described in Fig. 17.

C: Controls

B1: TGF-β1

FIGURE 20: Effect of TGF-β1 on TGF-β3 in astrocytes.

Astrocytes were incubated with TGF-β1 or TGF-β2 and c llular RNA was analyzed by Northern blot using

for hybridization a cRNA probe specific for TGF-β3. A single 3.5kb transcript is present in rat astrocytes.

FIGURE 21: Effect of TGF-β1 injection on NGF-mRNA in rat hippocampus.

TGF-β1 (5ng) was injected into the lateral brain ventricle of 8-day old rats. After 24 hours animals were killed by cervical dislocation and RNA from hippocampus was prepared and analyzed by Northern blots. The autoradiogram shows that the level of NGF-mRNA in hippocampus was increased by TGF-β1.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for regulating levels of nerve growth factor in the central nervous system, and is based on the discovery that in vivo synthesis of nerve growth factor may be regulated by various cytokines. According to the invention, regulation of NGF synthesis may be used to treat various central nervous system diseases and disorders.

For purposes of clarity, and not by way of limitation, the detailed description will be divided into the following sections:

(a) Identification of Cytokines Useful in Regulating NGF Synthesis
(b) Pharmaceutical Compositions and Administration
(c) Therapeutic Uses

5.1. IDENTIFICATION OF CYTOKINES USEFUL IN REGULATING NGF SYNTHESIS

The present invention relates to the use of cytokines which regulate NGF synthesis, including cytokines which increase NGF synthesis as well as cytokines which decrease NGF synthesis. In specific

embodiments of the invention, fibroblast growth factor (FGF, acidic or basic), platelet derived growth factor (PDGF), epidermal growth factor, interleukin 6, insulin-like growth factors 1 or 2, interferon gamma or tumor growth factor alpha (TGF-α) may be used to regulate NGF synthesis. In preferred embodiments of the invention, IL-1 or TGF-β may be used to increase NGF synthesis.

According to the invention, cytokines which may be tested for ability to regulate NGF synthesis include but are not limited to cytokines which have been identified in the central nervous system as well as cytokines which have not yet been associated with the central nervous system. Cytokines which may be tested for use include but are not limited to those listed in Table I, infra.

TABLE I
CYTOKINES

Symbol (Name)	Reference
Ciliary Neurotropic Factor	Hughes et al., 1988, Nature 335:70-73
Epidermal Growth Factor	Morrison et al., 1987, Science 238:72-75; Fallon et al., 1984, Science 224:1107-1109; Simpson et al., 1982, J. Neurosci. Res. 8:453-462
Fibroblast Growth Factor (Acidic)	Gimenez-Gallego et al., 1985, Science 230:1385-1388
Fibroblast Growth Factor (Basic)	Ferrara et al., 1988, Brain Res. 462:223-232
Endothelial Cell Growth Factor	Jaye et al., 1986, Science 233:541-545
Corticotropin Releasing	De Souza et al., 1984, Science 224:1449-1451
Growth Hormone Releasing Factor	Bloch et al., 1983, Nature 301:607-608
IL-1 (interleukin 1)	Henderson & Pettipher, 1988, Biochem. Pharmacol. 37:4171; Endo et al., 1988, BBRC 156:1007, Hopkins et al., 1988, Clin. Exp. Immunol. 72:422
IL-2 (interleukin 2)	Weil-Hillman et al., 1988, J. Biol. Response Mod. 7:424; Gemlo et al., 1988, Cancer Res. 48:5864
IL-3 (interleukin 3)	Schrader et al., 1986, Ann. Rev. Immunol. 4:205; Ziltener et al., 1988, Biol. Chem. 263:14511; Garnic : et al., 1988, Science 241:1820
IL-4 (interleukin 4)	Jankovic et al., 1988, Cell Immunol. 117:165; Broxmeyer et al., 1988, J. Immunol. 141:3852

IL-5 (interleukin 5)	Mita & Takatsu, 1988, Nippon Rinsho 46:1023; Beagley et al., 1988, J. Immunol. 141:2035
IL-6 (interleukin 6)	Ceuppens et al., 1988, J. Immunol. 141:3868; Suda et al., 1988, Exp. Hamatol. 16:891; Tovey et al., 1988, J. Immunol. 141:3106
IFNα (interferon α)	Pitha et al., 1988, J. Immunol. 141:3611; Mongini et al., 1988, Blood 72:1553
IFNγ (interferon γ)	Blanchard & Djeu, 1988, J. Immunol. 141:4067; Cleveland et al., 1988, J. Immunol. 141:3823
TRF (T cell replacing factor)	Hamaoka and Ono, 1986, Ann. Rev. Immunol. 4:167; Takatsu et al., 1988, Immunol. Rev. 102:107
TF (transfer factor)	Hancock et al., 1988, Eur. J. Cancer Clin. Oncol. 24:929
MIF (migration inhibition factor)	Malorny et al., 1988, Clin. Exp. Immunol. 71:164
LIF (leukocyte migration inhibitory factor	Szigeti & Rosen, 1988, Lymphokine Res. 7:11; Borish et al., 1988, Cell Immunol. 113:320
MFF (macrophage fusion factor)	Rodriguez Acosta et al., 1988, Scand. J. Immunol. 18:407
MAF (macrophage activation factor)	Watanabe et al., 1988, J. Biol. Response Mod., 6:556; Nakajima et al., Cancer Immunol. Immunother. 25:201
PAF (platelet activating factor)	Valone & Epstein, 1988, J. Immunol. 141:3945
TNFα (tumor necrosis factor α)	Plate et al., 1988, Ann. NY Acad. Sci. 532:149; Hopkins & Moager, 1988, Clin. Exp. Immunol. 73:88; Granger et al., 1988, J. Biol. Response Mod. 7:488
LT (lymphotoxin)	Shimoda et al., 1988, Cancer Immunol. Immunother. 26:101

IGF-1 (insulin-like growth factor-1)	Blundell and Humbel, 1980, Nature 287:781-787; Schoenle et al., 1982, Nature 296:252-253; Tannenbaum et al., 1983, Science 220:77-79; Dull et al, 1984, Nature 310:777-781; Bell et al., 1984, Nature 310:775-777
IGF-2 (insulin-like growth factor-2)	Blundell and Humbel, supra; Tannenbaum et al., 1983 (supra); Dull et al., 1984, (supra); Bell et al., 1984, (supra)
Insulin	Blundell and Humbel, supra
PDGF (platelet-derived growth factor)	Ross et al., 1986, Cell 46:155-169; Richardson et al., 1988, Cell 53:309-319
TGF-α (transforming growth factor -α)	Derynck, 1988, Cell 54:593-595
TGF-β (transforming growth factor -β)	Cheifetz et al., 1987, Cell 48:409-416
Bombesin	Rozengurt and Sinnett-Smith, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2936-2940; PNAS 80:2936-2940

In addition, substances which alter the levels of cytokines may be used according to the invention in order to indirectly alter levels of NGF. For example, and not by way of limitation, numerous native IL-1 inhibitors have been described (Larrick, 1989, Immunology Today 10:61-666, Table II); these IL-1 inhibitors may be used according to the invention to decrease levels of IL-1 and thereby decrease levels of NGF. Because these substances alter NGF levels by way of cytokines, their potential use is implicit in the present invention, which uses cytokines to regulate NGF levels. For example, glucocorticoids, which decrease IL-1 expression, may be used to decrease NGF levels in the central nervous system according to the invention (see Section 7, infra).

TABLE II

NATIVE IL-1 INHIBITORS

Source	
18-25kd protein from urine of monocytic leukemia patients	Seckinger et al., 1987, J. 39:1541-1545; Seckinger et al., 1987, J. Immunol. 139:1546-1549
30-35kd protein from urine of febrile patients	Liao et al., 1984, J. Exp. Med. 159:126-136; Liao et al., 1985, J. Immunol. 134:3882-3886
Uromodulin	Muchmore et al., 1986, J. Biol. Chem. 261:13404-13407; Muchmore and Decker, 1985, Science 229:479-481; Brown et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:9119-9123
Influenza or respiratory syncytial virus infected macrophages	Roberts et al., 1986, J. Exp. Med. 163:511-519
HIV-infected mononuclear phagocytes	Berman et al., 1987, Clin. Immunol. Immunopath. 42:133-140; Berman et al., 1986, Clin. Exp. Immunol. 64:136-145

PMN conditioned medium	Tiku et al., 1986, J. Immunol. 136:3686-3692.
Submandibular glands	Kemp et al., 1986, J. Immunol. 137:2245-2251
Glucocorticoid hormones	Bochner et al., 1987, J. Immunol. 139:2303-2307; Snyder and Unanue, 1982, J. Immunol. 129:1803-1810

In a preferred embodiment, both in vitro as well as in vivo testing are used to identify cytokines which are useful in regulating NGF synthesis; in vitro results may not reflect the degree to which a cytokine may regulate NGF synthesis in vivo (e.g. TGF-α and TGF-β stimulate NGF synthesis in purified astrocyte cultures to a greater degree than it stimulates NGF synthesis in vivo; see infra).

In vitro testing of cytokine ability to regulate NGF expression may be performed in astrocyte cultures which, preferably, contain greater than about 90 percent astrocytes and may be prepared by methods known in the art (see, e.g., section 6.1.2., infra). Cytokine may be inoculated into cultures at various concentrations and for various periods of time, in order to optimize the dose-response relationship for regulation of NGF production. NGF-mRNA may be measured by standard methods known to one skilled in the art, including in situ hybridization, quantitative "dot blot" or filter hybridization analysis, or quantitative Northern blot analysis. NGF levels may be measured by bioassay, by anti-NGF antibody binding in

situ, or by enzyme-linked immunosorbent assay (ELISA), among other standard methods.

Once suitable cytokines have been suggested by in vitro data, in vivo testing may be performed in suitable experimental animals, such as rats, with cytokine dosage spanning the optimal dosage determined by in vitro testing; dosages above and below the in vitro optimum may be tested. Cytokine, in suitable carrier, may be administered to test animals by intracerebroventricular injection according to standard methods. After a period of time, the animal may be sacrificed, and the brain excised for determination of NGF levels. NGF-mRNA may be measured by methods such as in situ hybridization, "dot-blot" or Northern blot analysis; NGF levels may be measured by bioassay, anti-NGF antibody binding in situ, or ELISA, among other standard techniques.

5.2. PHARMACEUTICAL COMPOSITIONS

The active compositions (i.e. cytokines, cytokine inhibitors, cytokine facilitating agents) of the invention may be administered in any sterile pharmaceutical carrier suitable for introduction into the central nervous system. The active compositions may be administered into the cerebrospinal fluid by any suitable route, including intraventricular as well as intrathecal injection, although intraventricular injection may be preferable in that it provides, albeit transiently, a higher local concentration. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. If an active composition is able to effectively cross the blood-brain barrier, then the composition may be administered intravenously.

5.3. THERAPEUTIC USES

The present invention, in which cytokines are used to regulate NGF synthesis, may be utilized to treat a variety of diseases and disorders of the central nervous system.

In various embodiments of the invention, cytokines may be used to regulate NGF synthesis in neurologic disorders including but not limited to those in which the central nervous system has been damaged by trauma, surgery, ischemia, infection, malignancy or toxic agents. The invention in particular may be used to treat conditions in which damage has occurred to the hippocampus, striatum, or basal forebrain.

In further embodiments of the invention, cytokines may be used to regulate NGF synthesis in congenital conditions, and, particularly, in congenital conditions which may be associated with deficiencies of NGF; thus the invention may be useful in certain congenital learning disorders.

In still further embodiments of the invention, cytokines may be used to regulate NGF synthesis in neurodegenerative disorders. In particular, cytokines may be used to treat dementias, such as Alzheimer's disease.

According to the invention, cytokines may be used to either increase or decrease NGF synthesis in the central nervous system. In a preferred embodiment of the invention, cytokine(s), such as IL-1, or TGF-β may be used to increase NGF levels in the central nervous system.

Non-cytokine substances which alter cytokine levels and thereby indirectly regulate NGF synthesis may also be used according to the invention.

In alternate embodiments, cytokine(s) may be used to decrease NGF levels in the central nervous system, utilizing, for example, substances which antagonize the

action of IL-1 (see Table II) including, but not limited to, glucocorticoid hormones; substances which decrease NGF synthesis may be useful in the treatment of conditions characterized by a relative predominance of CNS cholinergic activity, such as Parkinson's disease.

One of the advantages of the present invention is that it enables the targeting of NGF promoter activation and/or NGF expression to a particular area in need of such treatment. For example, by increasing the activity of endogenous CNS NGF production, NGF will be produced in an area which may benefit from increased levels of NGF, thereby sparing areas not normally exposed to NGF from potential adverse effects due to, for example, systemic NGF administration.

5.4. INDUCTION OF NGF PROMOTER ACTIVITY BY CYTOKINES

In alternative embodiments of the invention, the NGF promoter may be linked to a nucleic acid sequence encoding a protein or peptide of interest, and the transcription of the protein of interest may be controlled by exposing the NGF promoter to a substance which regulates the expression of NGF. The term "protein or peptide of interest" should be construed to refer to any peptide or protein that may be useful, therapeutically or diagnostically, and/or which has biological or chemical activity, including but not limited to, cytokines, hormone, enzymes, antibodies or antibody fragments, reporter or marker proteins, etc. In preferred embodiments of the invention, the peptide of interest may be a neurotrophic factor, including, but not limited to, NGF, BDNF, CNTF, NT-3, or a protein or peptide having about at least six amino acids which are homologous (i.e. are at least about 25 percent identical) to NGF, BDNF, CNTF, or NT-3. The present invention provides for a

variety of substances which regulate the expression of NGF (See Section 5.1., supra). Substances may either increase or, alternatively, decrease the activity of the NGF promoter.

The recombinant constructs of invention comprise at least a portion of the NGF promoter region which is responsive to an NGF-regulating substance. For example, and not by way of limitation, a useful region of the NGF promoter may be cloned substantially as set forth in Section 7.1.4., infra; basically, a Balb/c mouse genomic library may be screened with an oligonucleotide probe which spans the first exon of the NGF gene (Selby, M.J., et al., 1987, Mol. Cell. Biol. 7:3057-3064) to identify clones which comprise the NGF promoter region. An approximately 2.1kb HincII/PvuII fragment may be prepared from such a clone and then linked to nucleic acid sequence encoding a protein or peptide of interest. The resulting construct would preferably provide an appropriate initiation codon to create a translational reading frame which would produce the desired amino acid sequence. A ribosome binding site may also be included. In a specific embodiment of the invention, the HincII/PvuII fragment may be cloned into the XbaI/XhoI sites of the chloramphenicol acetyl transferase (CAT) bearing pBLCAT3 expression vector (Hengerer et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3899).

The NGF promoter-bearing recombinant nucleic acid constructs of the invention may be introduced into bacteria, yeast, or preferably, eukaryotic tissues, or cell lines, or, alternatively, may be incorporated as transgenes into transgenic animals or into organisms, including humans, by gene transfer (for example, via a viral vector). Introducti n of the constructs of the invention may be accomplish d by transfection,

transduction, calcium phosphate precipitation, electroporation, or cell gun. The present invention also provides for cells, tissues, cell lines, and transgenic animals which comprise the NGF promoter constructs of the invention.

The NGF promoter constructs may be utilized in systems to identify compounds which regulate NGF production. In preferred embodiments of the invention, the constructs may be used to identify compounds which regulate NGF production in the central nervous system. For example, and not by way of limitation, the construct of the invention may comprise the NGF promoter and the CAT gene, such that activation of the NGF promoter may result in increased CAT expression. This construct may be introduced into a suitable cell or cell line, such as cultured fibroblasts or astrocytes. The cells may then be exposed to a test agent; a subsequent increase in CAT activity may indicate activation of the NGF promoter by the test agent; alternatively, a subsequent decrease in CAT activity may indicate repression of NGF promoter activity by the test agent.

In further embodiments, the constructs of the invention may be used to create experimental systems for the study of central nervous system physiology. For example, and not by way of limitation, a non-human transgenic animal may be produced containing a construct comprising nucleotide sequence encoding a neurotrophic factor, such as BDNF. Administration of a substance which increases NGF promoter activity may result in increased levels of BDNF in the CNS of such an animal. The effects of BDNF, and the combination of BDNF and NGF, on such an animal may be ascertained by comparison with an animal lacking the construct but subjected to similar administration of NGF-inducing substance.

Introduction of NGF-bearing constructs into human subjects, for example by viral transduction, could be used in a similar manner to treat conditions which may favorably respond to production of a protein or peptide within the CNS. Induction of the NGF promoter of the constructs may be accomplished by exogenously administered or endogenous substances. For example, increased expression of a protein of interest may result from administration of a substance which increases NGF promoter activity into the CNS of a patient bearing a construct of the invention. Alternatively, expression of the protein or peptide of interest may be controlled by endogenous mechanisms. In Section 8.3., infra, it is stated that brain levels of TGF-β1 increase following brain injury. Therefore, in a patient suffering from repeated CNS insults, for example, multiple infarcts, the construct of the invention may comprise a peptide believed to enhance CNS repair, such as BDNF, CNTF, NT-3 or NGF, under the control of the TGF-β1 responsive NGF promoter. With each new insult, the brain of such a patient would respond with augmented self-repairing mechanisms. In an alternative specific embodiment of the invention, a patient suffering from Parkinson's disease may be treated by the transduction of a construct comprising the gene encoding tyrosine hydroxylase under the control of the NGF promoter.

6. EXAMPLE: COMPARISON OF THE EFFECTS OF INTERLEUKIN-1, TRANSFORMING GROWTH FACTOR-BETA AND VARIOUS OTHER GROWTH FACTORS ON NERVE GROWTH FACTOR (NGF) SYNTHESIS IN ASTROCYTE CULTURES AND IN VIVO

6.1. MATERIALS AND METHODS

6.1.1. ANTIBODIES AND CYTOKINES

EGF was obtained from Sigma, PDGF from Biochrom and bovine basic FGF from Amersham. Anti-Thy 1.1 antibody was obtained from Serotec and anti-GFAP and anti-vimentin were obtained from Boehringer Mannheim. 04-Antibody was a gift of Dr. I. Sommer, IL-1-β was a gift of Dr. A. Gronenborn, and TGF-α was a gift of Dr. W. Risau. The NGF-antibody-galactosidase conjugate for the NGF-ELISA and its substrate chlorophenol-red-β-D-galactopyranoside were obtained from Boehringer Mannheim; TGF-β1 was obtained from R&D Systems, Inc., MN.

6.1.2. CELL CULTURES

DME-medium and penicillin-streptomycin solution were obtained from GIBCO. Fetal calf serum was obtained from Boehringer Mannheim. Primary cultures were prepared from neonatal rat brain according to McCarthy and de Vellis (1980, J. Cell. Biol. 85:890-902). The cells were kept in DMEM supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin and grown in 10% CO_2/90% air at 37°C. Medium was changed every fourth day. The cells were confluent after 2-3 weeks.

Purified astrocyte cultures were obtained by the following procedure:
Oligodendrocytes and microglial cells growing on top of the astrocyte monolayer were removed by shaking the culture flask on a rotary shaker overnight at 180 rpm at 37°C. Detached cells were removed. The cultures were then subjected to 20 mM EDTA in PBS for 15-20 minutes. The resulting cell suspension was centrifuged and resuspended in DMEM with 10% FCS. The next purification step is based on the fact that, after replating, microglial cells attach much faster to uncoated tissue culture plastic than astrocytes. After 30 minutes, unattached astrocytes were taken off and finally plated on poly-L-lysine coated tissue culture dishes

(60 x 15 mm). They were maintained in DMEM with 10% FCS until they were confluent. For investigating the effects of IL-1 and various growth factors on NGF synthesis, the cultures were kept in medium containing 0.5% FCS for 24 hours (see results) and the following compounds were added for 4 and 24 hours: IL-1β (0.75 and 7.5 ng/ml), EGF (5 ng/ml), bFGF (5 ng/ml), and TGF-α (10 ng/ml). Cultures were exposed to TGF-β at a concentration of 5 ng/ml for a total of 48 hours.

6.1.3. IDENTIFICATION OF INDIVIDUAL GLIAL CELL POPULATIONS

The cells were characterized by immunocytochemistry. After fixation and permeabilization in 5% acetic acid/95% ethanol at -20°C and preincubation with 1% BSA, mouse monoclonal antibodies against GFAP and vimentin (Dahl, 1981, J. Neurosci. Res. 6:741-748) were incubated for 1 hour at room temperature. O4, a mouse monoclonal antibody reacting with a cell surface constituent of young oligodendrocytes (Sommer and Schachner, 1981, Dev. Biol. 83:311-327), and anti-Thy 1.1, a mouse monoclonal antibody, which was used to identify fibroblasts (Raff et al., 1979, Brain Res. 174:283-308), were incubated with cells in culture for 1 hour, after which these were fixed in 4% paraformaldehyde. All cells were incubated for 30 minutes at room temperature with fluorescein-labeled sheep anti-mouse immunoglobulins (Gibco), diluted 1:100, as a second antibody. The cultures were examined using a Zeiss fluorescence microscope.

Microglial cells were identified by their nonspecific esterase reaction according to Koski et al. (1976, in In Vitro Methods in Cell Mediated and Tumor Immunity, Bloom and David, eds., Academic Press, New York, p. 359). Briefly, the cells were fixed with 4%

paraformaldehyde and incubated with pararosaniline and alpha-naphthylacetate. After 10-20 minutes the dark red-brown precipitate became visible by light microscopy.

6.1.4. IN SITU HYBRIDIZATION

In order to determine which cells express NGF-mRNA, in situ hybridization experiments on mixed primary rat brain cultures were performed according to Bandtlow et al. (1987, EMBO J. 6:891-899).

6.1.5. NORTHERN BLOT ANALYSIS

Quantitative Northern blot analysis was performed according to Heumann and Thoenen (1986, J. Biol. Chem. 261:9246-9249). For absolute quantification, a recovery standard (510 b) and a calibration standard (910 b) were coelectrophoresed in separate lanes (Heumann and Thoenen, 1986, supra; Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351). Hybridization was carried out at 65°C with 5 x 10^6 cpm/ml of ^{32}P-labeled NGF-cRNA-probe (0.9 kb) in 50% formamide.

6.1.6. NGF-ELISA

For measurement of NGF in supernatant, 0.1% BSA was added with the tested compounds. After 24 hours the medium was removed and two site enzyme linked immunoassay was performed. The medium was incubated with 27/21 anti-NGF monoclonal antibody, which was coated onto Nunc Immuno Quality 1 multititer plates. NGF was detected by the same antibody coupled to β-D-galactosidase. Substrate of the enzymatic reaction was chlorophenol red-β-D-galactosidase (CPRG); absorbance was measured at 550 nm.

6.1.7. IN VIVO EXPERIMENTS

The in vivo experiments were performed using 10 day old Wistar rats. After anaesthesizing the animals with ether, 15 ng IL-1-β, 20 ng bFGF, 20 ng TGF-α 5 ng TGFβ_1 or 4 μl PBS as control were injected intracerebroventricularly. Eight hours later hippocampus and cortex were removed for NGF-mRNA determination.

6.2. RESULTS

6.2.1. PURITY OF CULTURES EVAULATED BY IMMUNOHISTOCHEMICAL PROCEDURES

Primary cultures of brain cells from newborn rats were grown in DMEM supplemented with 10% fetal calf serum until they were confluent. After purification (see methods) secondary cultures consisted of about 95% astrocytes, which all contained vimentin, but were only 70% GFAP (Fig. 1). This observation is in agreement with Dahl (1981, J. Neurosci. Res. 6:741-748), who showed that immature astrocytes are $GFAP^-$. Contaminating cells were 1-2% $Thy\text{-}1^+$ fibroblasts, and 3-4% 04^+ oligodendrocytes (Fig. 2a). Very few microglial cells were present, as shown by staining for unspecific esterase (Fig. 2c). Neurons were not present in these cultures.

6.2.2. IN SITU HYBRIDIZATION OF CULTURES

In situ hybridization of mixed rat brain primary cultures revealed that oligodendrocytes and microglia do not express NGF-mRNA. Specific labeling was confined to astrocytes. The signal over astrocytes in unstimulated cultures was very weak, but increased markedly after stimulation with serum or TGF-α. Oligodendrocytes and microglial cells remained unlabeled (Fig. 3, arrow). For technical reasons it was not possible to combine in situ hybridization and immunochemistry in the same preparations.

6.2.3. EFFECT OF FETAL CALF SERUM ON NGF SYNTHESIS

In preliminary experiments we found an induction of NGF-mRNA expression and NGF-synthesis by FCS. After adding 10% FCS, NGF and its mRNA were increased for at least 48 hours (Fig. 4). This observation is in agreement with that of Furukawa et al. (1987, Biochem. Biophys. Res. Commun. 142:395-402), who demonstrated increased NGF-levels in the culture medium after addition of serum. Since serum contains various potential regulatory molecules for NGF synthesis such as PDGF and EGF, the cells were kept in medium containing only 0.5% FCS. This low concentration had no measurable effect on proliferation or NGF-mRNA expression, but was necessary to keep the cells in good condition.

6.2.4. EFFECT OF cAMP AND NOREPINEPHRINE ON NGF SYNTHESIS IN VITRO

Norepinephrine reduces NGF-synthesis in the periphery, i.e. rat iris cultures (Hellweg et al., 1988, Exp. Cell. Res. 179:18-30). In contrast, cAMP was reported to increase NGF-mRNA levels in C6-Glioma cells (Schwartz, 1988, Glia 1:282-285). Under our experimental conditions we did not observe a measurable change in NGF-mRNA levels by 8-bromo-cAMP, nor by norepinephrine (Fig. 5).

6.2.5. EFFECT OF IL-1, TGF-β AND OTHER GROWTH FACTORS ON NGF SYNTHESIS IN VITRO

IL-1β and a series of growth factors which have been reported to be present in rodent brain, as demonstrated by immunoassays, immunohistochemistry and in situ hybridization (Wilcox and Derynck, 1988, J. Neurosci. 8:1901-1904; Fallon et al., 1984, Science 224:1107-1109; Giulian and Lachman, 1985, Science 228:497-499; Gospodarowicz, 1987, Endocrine Reviews 8:95-116), were

tested for their effects on NGF synthesis. They were used in saturating concentrations, i.e. they were supramaximal with respect to their reported mitogenic action (Giulian et al., 1986, J.Exp. Med. 164:594-604; Gospodarowicz et al., 1987, Endocrine Reviews 8:95-116; Avola et al., 1988, J. Neurosci. Res. 19:230-238; Noble et al., 1988, Nature 333:560-562). Moreover, preliminary experiments with high concentrations used in single experiments did not further increase NGF-mRNA levels.

IL-1β increased NGF-mRNA about 5-fold, whereas bFGF and EGF increased it about 7-fold. TGF-α had a strong effect and increased NGF-mRNA about 9-fold as determined by quantitative Northern blot analysis (Fig. 5). Maximal NGF-mRNA levels were reached after 6 hours incubation with 7.5 ng IL-1β and then declined gradually, approaching control levels after 24 hours (Fig. 6). We assumed a similar time course for TGF-α, bFGF and EGF, since the relative changes of NGF-mRNA after 6 and 24 hours were similar to those in the presence of IL-1β.

Both TGF-β1 and TGF-β2 were observed to strongly stimulate NGF-mRNA levels in rat astrocytes in a time and concentration-dependent manner (Figures 7 and 8). The maximal effect of TGF-β (a 50-fold increase in NGF-mRNA) was seen at 24 hours after addition, in contrast to the situation found with other growth factors, including IL-1β, which achieved maximal NGF mRNA levels after 6 hours in culture and induced relatively lower levels of NGF mRNA.

NGF accumulating in the culture medium during 24 hours incubation with IL-1β and growth factors was measured by ELISA. IL-1β, EGF, TGF-α, bFGF and PDGF all increased NGF concentration in the medium (Fig. 9). The absolute quantities measured could be increased 2-3-fold by adding protease-inhibitors (11 mM leupeptin and 40 U aprotinin).

However, the relative amounts of NGF released reflected the increase in NGF-mRNA.

6.2.6. EFFECTS OF TGF-β, IL-1β AND OTHER GROWTH FACTORS ON NGF SYNTHESIS IN VIVO

In order to verify the physiological significance of the in vitro results, TGF-α, FGF, TGF-β and IL-1β were injected into the lateral ventricles of 10 day old rat brains in pharmacological concentrations. Whereas 20 ng of both TGF-α and FGF had only a small inducing effect, 16 ng of IL-1β increased NGF-mRNA in the hippocampus 4-5-fold and 5 ng of TGF-β1 increased the NGF-mRNA level in the hippocampus 3 to 4-fold (Fig. 10). Neither IL-1β nor FGF produced measurable changes in NGF-mRNA levels in the neocortex.

6.3. DISCUSSION

The goal of the present study was to obtain information on the mechanisms involved in the regulation of NGF synthesis in the CNS. After we had demonstrated that in mixed and purified cultures of astrocytes, oligodendrocytes, and microglial cells, NGF synthesis took place only in astrocytes, we studied the effect of TGF-β, IL-1 and a series of growth factors in highly purified rat astrocyte cultures.

The selection of the growth factors and IL-1 was based on the evidence for their synthesis in the brain. EGF was included because TGF-α may act via EGF receptors. These receptors have been demonstrated to be present by immunohistochemical procedures on neurons of various brain regions (Gomez-Pinilla et al., 1988, Brain Res. 438:385-390) and on astrocytes for which the immunoreactivity was enhanced after brain lesion (Nietro-Sampedro et al., 1988, Neurosci. Lett. 91:276-282). Both for IL-1 and all the

growth factors investigated, the concentrations used were supramaximal. After preliminary experiments had demonstrated that the maximal effect for all growth factors tested (except for TGF-β) was reached after 4-6 hours, we restricted our experiments to 6 and 24 hours after the addition of the individual factors, with the exception of IL-1, for which a full time-course was established. The effects on NGF-mRNA levels after 6 hours varied between 5-fold increase for IL-1 and a 9-fold increase for TGF-α. With the exception of TGF-β, for all molecules investigated the levels of NGF-mRNA approached control levels after 24 hours. In order to decide whether the decay of NGF-mRNA between 6 and 24 hours was due to a desensitization of the corresponding receptor-transducing systems or due to a degradation of the polypeptides added, we readded in three experiments IL-1, TGFα and FGF after 24 hours and observed a reincrease of the NGF-mRNA levels. Thus, an inactivation of the added molecules seems to be the predominant cause for the fading effect on NGF-mRNA. This is in agreement with previous observations made with IL-1 in cultures of rat sciatic nerves (Lindholm et al., 1987, Nature 330:658-659).

In contrast to IL-1 and the other growth factors tested, TGF-β1 elicited maximal levels of NGF mRNA after 24 hours of culture; furthermore, high levels of NGF mRNA were maintained for at least 48 hours of culture. As shown in Figure 10, 5 ng/ml of TGF-β was associated with maximal induction of NGF mRNA. We have also observed that the TGF-β mediated increase in NGF-mRNA could be blocked with cycloheximide (data not shown), and is most probably due to transcriptional enhancement of the NGF gene. Interestingly, Northern blot analysis has demonstrated that TGF-β1 is expressed in many areas of adult rat brain.

The increase in NGF-mRNA was reflected by a corresp nding increase in NGF protein released int the

culture medium. This observation is in agreement with previous, more detailed experiments with primary cultures of rat iris in which the increase in NGF-mRNA was followed by a corresponding increase in synthesis and release of NGF protein (Heumann and Thoenen, 1986, J. Biol. Chem. 261:9246-9249). In contrast, neither norepinephrine nor 8-bromo-cAMP changed NGF-mRNA levels in our astrocyte cultures. Previously it has been demonstrated that norepinephrine resulted in a marked decrease in NGF and NGF-mRNA levels in organ cultures of rat irises (Hellweg et al., 1988, Exp. Cell. Res. 179:18-30); in addition, an increase in NGF-mRNA by both norepinephrine and 8-bromo-cAMP has been reported for C6 glioma cells (Schwartz, 1988, Glia. 1:282-285).

Based on the results of the in vitro experiments in astrocyte cultures, in vivo experiments have been performed, i.e. IL-1, TGF-β EGF, and TGF-α were injected intraventricularly into rat brains. The results obtained in vivo were not fully reflective of the observations made in astrocyte cultures; for example, the injection of TGF-α, which produced a significant effect in astrocyte cultures on NGF-mRNA was without detectable effect in vivo (it cannot be decided whether the discrepancy between the in vivo effects of TGF-α is due to its rapid degradation after intraventricular injection or that the responsiveness of astrocytes in culture is different from that in situ). However, in contrast to TGF-α, the effects of TGF-β and FGF on cultured astrocytes were also reflected by an increase in NGF-mRNA after intraventricular injection, although the effects in vivo were smaller than those observed in astrocyte cultures. The observation that FGF increases NGF-mRNA both in vitro and in vivo is of particular interest in view of the observation that after fimbria lesion, infusi n of FGF prevents the degeneration of septal

cholinergic neurons in a similar manner as originally observed by Hefti after NGF injection (1986, Exp. Cell. Res. 179:18-30). Since the direct responsiveness of these neurons to NGF is firmly established (see Thoenen et al., 1987, Rev. Physiol. Biochem. Pharmacol. 109:145-178; Whittemore and Seiger, 1987, Brain Res. Rev. 12:439-464) and since the present experiments have demonstrated that FGF enhances the synthesis of NGF not only in vitro, but also in vivo, it would seem that at least part of the observed protecting effect of FGF on septal cholinergic neurons is an indirect one. Of particular interest is the marked effect of IL-1 on NGF-mRNA levels in vivo, whereas in astrocyte cultures it was in the range of that of FGF, but distinctly smaller than that of TGF-β, TGF-α and EGF. Thus, the question arises whether the effect of IL-1 in the brain results not only from the regulation of NGF synthesis in astrocytes but also involves NGF synthesis in other cells of the brain. Indeed, pyramidal neurons of the hippocampus and granule cells of the nucleus dentatus have been shown to express IL-1 receptors; in recent in situ experiments it has been shown that the same neurons also express NGF-mRNA (Rennert and Heinrich, 1986, Biochem. Biophs. Res. Commun. 138:813-818; Ayer-LeLievre et al., 1988, Science 240:1339-1341; Whittemore et al., 1988, J. Neurosci. Res. 20:403-410). Thus, the relatively large effect of IL-1 in vivo as compared to its effect on astrocyte cultures could result from a regulatory function of IL-1 on the NGF synthesis in these neurons of the CNS. An additional interesting aspect concerns the relatively large effects of IL-1 on NGF-mRNA in vivo. Pyramidal cells of the hippocampus and granule cells of the gyrus dentatus express IL-1 receptors and NGF-mRNAs as well as IL-1β-mRNA (Bandtlow et al., 1987, EMBO J. 6:891-899). The fact that IL-1 receptor and NGF mRNAs are

present in the same neurons as IL-1 mRNA suggests that IL-1 levels may be regulated in an autocrine manner.

Autocrine regulation of IL-1 in the hippocampus is suggested by preliminary observations demonstrating that after intraventricular injection of IL-1 there is not only an increase in NGF-mRNA but also a substantial increase in IL-1β-mRNA. This, together with the differential localization of IL-1β-mRNA and IL-1 activity, suggests that IL-1 might play a role in the regulation of NGF synthesis in both the central nervous system as well as the periphery.

7. EXAMPLE: GLUCOCORTICOID HORMONES NEGATIVELY REGULATE NERVE GROWTH FACTOR EXPRESSION

Sciatic nerve transection leads to an up-regulation of nerve growth factor (NGF) production in non-neuronal cells surrounding the axons. The lesion-mediated increase in NGF-mRNA levels in the nerve can be blocked by pretreating the animals with the synthetic glucocorticoid, dexamethasone. Dexamethasone also reduces NGF-mRNA levels in cultured sciatic fibroblasts and astrocytes stimulated with fetal calf serum or interleukin-1 (IL-1). To study at which level glucocorticoids down-regulate NGF expression, sciatic fibroblasts where transfected with a construct containing the NGF promoter and the chloramphenicol acetyltransferase (CAT) reporter gene. The results indicate that dexamethasone effectively represses NGF gene transcription and thus inhibits the production of this neurotropic molecule.

7.1. MATERIALS AND METHODS

7.1.1. SCIATIC NERVE TRANSECTION

Wistar rats (160-180 g) of both sexes were used throughout this study. The rats were anesthesized and the sciatic n rve was cut unilaterally with fine scissors at the

sciatic notch (Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H., 1987, J. Cell. Biol. 104:1623-1631). After 6 hours the animals were killed by decapitation and 0.5 cm and 1 cm segments of the nerve, proximal and distal to the cut site were removed and immediately frozen on dry ice. In some experiments rats were pretreated with dexamethasone (2.5 mg/kg body weight) which was injected intraperitoneally 2 hours before nerve lesion. Control rats were injected with an equal volume of 0.9% NaCl.

7.1.2. CELL AND ORGAN CULTURE

Sciatic nerve segments (3 cm) were prepared and placed into culture in 1 ml of Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum. In order to study the effect of serum, some cultures were incubated without serum supplementation. The incubation was carried out for 6 hours and dexamethasone (1 μM) was present where indicated in the figures.

Rat sciatic fibroblasts were prepared and cultured as previously described (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). Confluent cells were kept in low (1%) fetal calf serum and stimulated with IL-1 (30 units/ml) or with 10% fetal calf serum. Dexamethasone (1 μM) was added to some cultures.

Astrocytes were prepared from neonatal rat brain according to McCarthy and de Vellis (1980, J. Cell. Biol. 85:890-902).

7.1.3. RNA PREPARATION AND NORTHERN BLOT ANALYSIS

Total RNA was prepared from fibroblasts and frozen nerves as described earlier (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351; Lindholm, D., Heumann, R., Meyer, M., and

Thoenen, H., 1987, Nature 330:658-659). In all experiments a shortened NGF standard (0.52 kb) was included to estimate RNA recovery. RNA samples were glyoxylated and electrophoresed through a 1.5% agarose gel and a 0.92 kb NGF transcript was co-electrophoresed in a separate lane to allow quantification of the autoradiograms (Lindholm, D., Heumann,R., Meyer, M., and Thoenen, H., 1987, Nature 330:658-659; Heumann, R., and Thoenen, H., 1986, J. Biol. Chem. 261:9246-9249). Following electrophoresis, RNA was transferred to nylon membranes which had been UV-irradiated, prehybridized and hybridized as previously described (Heumann, R., and Thoenen, H., 1986, J. Biol. Chem. 261:9246-9249). The NGF cRNA probe was prepared using the riboprobe system and a Pst I-derived fragment of mouse NGF-cDNA (Heumann, R., and Thoenen, H., 1986, J. Biol. Chem. 261:9246-9249). After hybridization, the filters were washed and exposed to x-ray films. The quantification of NGF-mRNA levels were done by scanning of the autoradiograms on a LKB laser scanner.

7.1.4. DNA CONSTRUCTION AND TRANSFECTION

For cloning of the NGF promoter, a Balb/c mouse genomic library was screened with a 33 mer oligonucleotide spanning the first exon of the NGF gene (Selby, M. J., Edwards, R., Sharp, F., and Rutter, W. J., 1987, Mol. Cell. Biol. 7:3057-3064). A 2.1 kb HincII/PvuII fragment of the promoter region was cloned in to the XbaI/XhoI sites of the CAT-expression vector pBLCAT 3 and the gene construct (pBLPNCAT3) was used for transfection.

Two days after replating, rat sciatic fibroblasts (80-90% confluent) were transfected for 14 hours with 10 µg of pBLPNCAT3 using a modification of the calcium phosphate method. F llowing washing, the cells were incubated for 24 hours in DMEM alone or with either 10% fetal calf serum or

IL-1. Dexamethasone (1 μM) was present where indicated in Figure 14. The cells were harvested and equal amounts of proteins in the extracts were used to measure CAT activity.

7.1.5. DETERMINATION OF CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ACTIVITY

Chloramphenicol acetyl transferase (CAT) activity was determined according to the method of Gorman et al. (1982, Mol. Cell. Biol. 2:1044-1051).

7.1.6. REAGENTS

Recombinant human IL-1 was a generous gift from Dr. A. Gronenborn, Martinsried. Dexamethasone was obtained from Sigma Chemical Co.

7.2. RESULTS

7.2.1. EFFECT OF DEXAMETHASONE ON THE LESION-MEDIATED INCREASE IN SCIATIC NGF-mRNA

In previous experiments we have shown that sciatic nerve lesion stimulates the synthesis of NGF in nerve segments proximally and distally to the cut site (Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H., 1987, J. Cell. Biol. 104:1623-1631; Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E., and Thoenen, H., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:8735-8739). In addition, the initial increase in sciatic NGF-mRNA occurs also when sciatic nerve segments are brought into culture (Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E., and Thoenen, H., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:8735-8739). However, as shown in Figure 11, the lesion-mediated increase in sciatic NGF-mRNA levels could be totally blocked by pretreating the animals with dexamethasone. Usually, 2.5 mg/kg body weight of the steroid were injected but the

effect was also observed with 1 mg dexamethasone/kg. Although dexamethasone was very effective in suppressing the increased NGF-mRNA levels after lesion, dexamethasone did not seem to affect the low basal levels of NGF-mRNA in the intact sciatic nerve (Figure 11). Dexamethasone also reduced NGF-mRNA levels in sciatic nerve segments incubated in vitro (Figure 11). However, the degree of inhibition of NGF-mRNA increase in vitro by dexamethasone was not complete; it was reduced by about 80% for nerves incubated either in the absence or presence of serum (see Figure 11). Figure 11 also shows that the increase in NGF-mRNA was lower following transection in vivo as compared to that occurring in cultured nerve segments. This is in keeping with previous observations (Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E., and Thoenen, H., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:8735-8739) and could possibly be due to the action of endogenous glucocorticoids released during the stress response following nerve lesions. Indeed, preliminary experiments indicated that the increase in sciatic NGF-mRNA levels after sciatic nerve lesion is higher in adrenalectomized rats as compared to control animals. Interestingly, regarding cultured nerve segments under various experimental conditions, we observed that the increase in sciatic NGF-mRNA levels was greater in the presence than in the absence of fetal calf serum (Figure 11). This indicates that factors present in serum contribute to the elevation of NGF-mRNA observed in cultured sciatic nerve segments.

7.2.2. REDUCTION OF NGF-mRNA LEVELS IN FIBROBLASTS AND ASTROCYTES BY DEXAMETHASONE

In order to investigate whether dexamethasone is equally effective in interfering with NGF-mRNA levels in diss ciated cells, fibroblasts were prepared from rat

sciatic nerves (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). As shown in Figure 12, dexamethasone reduced NGF-mRNA levels in cells treated with either serum or IL-1. The degree of inhibition of NGF expression by dexamethasone was about 70% in these cultures (Figure 12). Moreover, the low levels of NGF-mRNA in control cells were also further reduced by dexamethasone treatment (Figure 12, lane 2).

Figure 13 shows that dexamethasone rapidly reduced the accumulation of NGF-mRNA in the stimulated fibroblasts. Thus, dexamethasone added 1 or 2 hours after serum was still able to inhibit (by about 70%) the rise in NGF-mRNA induced by serum (lanes 4 and 5). However, adding the steroid 3 hours after serum (1 hour before incubation stop) only slightly (by 25%) reduced the level of NGF-mRNA (Figure 13, lane 6). In addition, cycloheximide, which inhibits protein synthesis, did not reduce the effect of dexamethasone in these cultures.

Moreover, as shown in Figure 13 (lane 7), 10 nM of dexamethasone also effectively inhibited NGF expression in these cells. In other experiments we found that even 0.1 nM of the steroid still had an inhibitory effect in this system.

To determine whether dexamethasone also inhibits NGF expression in astrocytes, the cultures were treated with TGFβ_1 (5 ng/ml) and/or dexamethasone (1 μM). The drug was found to suppress (more than 50%) the TGFβ_1-mediated increase in NGF-mRNA levels.

7.2.3. EFFECT OF DEXAMETHASONE ON NGF PROMOTER ACTIVITY

In order to decide whether dexamethasone interferes with NGF gene activity, sciatic fibroblasts were transfected with a vector construct containing the NGF

promoter linked to a CAT reporter gene. The addition of serum to these cells lead to an increase in CAT activity reflecting an enhanced NGF promoter activity which was markedly reduced by dexamethasone (Figure 14 a). Figure 14 b summarizes the results obtained with the transfected cells treated with serum and/or dexamethasone; the steroid was found to completely block the serum-mediated increase in CAT activity in these cells. Moreover, dexamethasone also suppressed (by about 40%) the low basic NGF gene activity in untreated, control fibroblasts (Figure 14 b). Experiments performed using IL-1 stimulated fibroblasts revealed that dexamethasone also counteracts the IL-1 induced increase in NGF gene activity. These results show that dexamethasone effectively regulates NGF-mRNA expression at the gene level.

7.3. DISCUSSION

Glucocorticoid hormones are known to stimulate (Yamamoto, K. R., 1985, Annu. Rev. Genet. 19:209-252) or inhibit (Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D., and Mellon, P.L., 1988, Science 241:350-353) the expression of specific genes with many physiological consequences. In particular, glucocorticoids act as important anti-inflammatory and immunomodulatory agents in man and in experimental animals. For example, glucocorticoids induce the production of the protein lipocortin-1 (Flower, R. J., and Blackwell, G. J., 1979, Nature 178:456-459) which results in an inhibition of phospholipase A2 activity and leads to a decrease in the production of inflammatory mediators, such as prostaglandins and leukotriene. Conversely, the lymphokine, interleukin-1 (IL-1) (Dinarello, C. A., 1984), Rev. Infect. Dis. 6:51-87), is known to enhance the release of prostaglandins (Dayer, J.-M., Goldring, S.R., R binson, D. R., and Krane, S. M., 1979, Biochem. Biophys. Acta 586:87-105) and t activate

phospholipase A2 (Chang, J. Gilman, S., and Lewis, A. J., 1986, J. Immunol. 136:1283-1287). Thus, in many systems glucocorticoids counteract the effects of IL-1 and also suppress the release of IL-1 from activated macrophages (Snyder, D. S., and Unanue, E. R., 1982, J. Immunol. 129:1803-1805).

We have recently shown that dexamethasone (dexa) inhibits the IL-1-mediated increase in NGF-mRNA levels in cultured sciatic fibroblasts (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). However, the mechanism for this suppressive effect of dexamethasone on NGF-mRNA was unclear and was thought to predominantly involve the inhibition of phospholipase A2 by lipocortin which is induced by dexamethasone (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). Moreover, it has recently also been reported that glucocorticoids rapidly decrease NGF-mRNA levels in mouse L 929 cells (Winon, D., Houlgatte, R., and Brachet, P., 1986, Exp. Cell Res. 162:562-565; Siminoski, K., Murphy, R. A., Rennert, P., and Heinrich, G., 1987, Endocrinology 121:1432-1437). In the present study we have, in more detail, studied the mechanism by which glucocorticoid hormones inhibit NGF expression. The results indicate that treatment with dexamethasone results in a reduction of NGF-mRNA levels in the sciatic nerve both in vivo and in vitro. Moreover, as shown in transfection experiments, the effect of dexamethasone is mainly due to an inhibition of NGF transcription.

In this study we demonstrate that glucocorticoid hormones are potent regulators of NGF expression, decreasing the level of NGF-mRNA both in vivo and in vitro. Thus, dexamethasone was found to attenuate the lesion-mediated increase in NGF-mRNA in rat sciatic nerve and to decrease

the level of NGF-mRNA in cultured sciatic fibroblasts. Furthermore, dexamethasone was found to inhibit NGF expression in astrocytes, having been observed to suppress the increase in NGF-mRNA levels. The results of transfection studies with the NGF-CAT gene construct showed that dexamethasone effectively inhibits transcription of the NGF gene. Moreover, the results showed that the sequences required for glucocorticoid hormone-mediated inhibition of NGF transcription reside within 2.1 kb of the NGF promoter (see below).

We have previously reported that IL-1 can increase the stability and enhance the transcription of NGF-mRNA in cultured fibroblasts (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). As shown here, dexamethasone is able to partly block the IL-1-mediated increase in NGF-mRNA in these cells. In addition, dexamethasone was also shown to inhibit the transcriptional activation of the NGF gene by IL-1 using the transfected NGF-CAT gene construct. Previously we had observed that a long-term (20 hours) pretreatment with dexamethasone completely inhibited the IL-1-mediated increase in fibroblast NGF-mRNA (Lindholm, D., Heumann, R., Hengerer, B., and Thoenen, H., 1988, J. Biol. Chem. 263:16348-16351). As shown in Figure 12, the reduction of NGF-mRNA in the fibroblasts after 3 hours with dexamethasone was about 70%, suggesting that a long-term steroid treatment is even more effective in inhibiting NGF expression.

Dexamethasone also reduced the increased NGF-mRNA levels observed after serum-treatment of cultured fibroblasts. Serum has earlier been shown to increase NGF synthesis in mouse L-cells (Wion, D., Houlgatte, R., Barbot, N., Barrand, P., Dicou, E., and Brachet, P., 1987, Biochem. Biophys. R s. Commun. 149:510-514) and rat astrocytes (Furukawa, S., Furukawa, Y., Satoyoshi, E., and Hayashi, K.,

1987, Biochem. Biophys. Res. Commun. 142:395-402). We found that the effect of serum depends on RNA synthesis actinomycin-D-sensitive) and involves transcription as demonstrated by the CAT reporter gene (Figure 4). Serum contains a number of different growth factors and the various combinations were not further analyzed here. However, we have earlier shown that platelet derived-growth factor increases NGF expression in the rat sciatic nerve (Lindholm, D., Heumann, R., Meyer, M., and Thoenen, H., 1987, Nature 330:658-659).

Previous studies have shown that glucocorticoid hormones can positively regulate transcription of various genes including murine mammary tumor virus (Chandler, V. L., Mater, B. A., and Yamamoto, K. R., 1983, Cell. 33:489-499) and metallothionein (Karin, M., Haslinger, A., Holtgreve, A., Richards, R. J., Krautor, P., Westphal, H. M., and Beato, M., 1984, Nature 308:513-519). Molecular studies indicate that the glucocorticoid-receptor complex binds to specific DNA sequences, termed glucocorticoid-responsive-elements (GRE) which act as enhancers in the glucocorticoid modulation of transcription (Yamamoto, K. R., 1985, Annu. Rev. Genet. 19:209-252; Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J.-A., Tsai, M.-J., and O'Malley, B. W., 1988, Cell 55:361-369. However, glucocorticoids can also influence post-transcriptional events, and for instance the hormones have been shown to increase the stability of mRNA encoding growth hormone (Paek, I., and Axel, R., 1987, Mol. Cell. Biol. 7:1496-1507), but glucocorticoids can also enhance the transcription of this gene (Slater, E. P., Rabenau, O., Karin, M., Baxter, D., and Beato, M., 1985, Mol. Cell. Biol. 5:2984-2992.a). Likewise, glucocorticoids are known to inhibit the release of IL-1 by activated macrophages and monocytes (Snyder, D. S., and Unanu , E. R., 1982, J.

Immunol. 129:1803-1805), by mechanisms most probably involving both the inhibition of transcription of the IL-1a gene and a decrease in the stability of IL-1-mRNA (Slater, E. P., Rabenau, O., Karin, M., Baxter, D., and Beato, M., 1985, Mol. Cell. Biol. 5:2984-2992.a; Lee, S. W., Tsou, A.-P., Chan, H., Thomas, J., Petrie, K., Eugui, E. M., and Allison, A., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:1204-1208). Moreover, glucocorticoids were also reported to inhibit the transcription of the metalloprotease, stromelysin (Frisch, S. M., and Ruley, H. E., 1987, J. Biol. Chem. 34:16300-16304) and the genes for collagen types IU and IV (Weiner, F. R., Czaja, M. J., Jefferson, D. M., Giambrone, M. A., Tur-Kaspa, R., Reid, L. M., and Zern, M., 1987, J. Biol. Chem. 262:6955-6958).

It has recently been shown that the glucocorticoid-receptor complex inhibits the transcription of the human glycoprotein hormone alfa-subunit probably by interfering with the binding of some positively acting transcription factors (Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D., and Mellon, P.L., 1988, Science 241:350-353). In the present study we demonstrate that glucocorticoids down-regulate expression of NGF in fibroblasts mainly by inhibiting transcription.

8. EXAMPLE: TRANSFORMING GROWTH FACTOR-β1 STIMULATES EXPRESSION OF NERVE GROWTH FACTOR IN THE RAT CENTRAL NERVOUS SYSTEM

8.1. MATERIALS AND METHODS

8.1.1. MATERIALS

Porcine platelet TGF-β1 and neutralizing anti-TGF-β-antibodies were obtained from R&D Systems Inc.

8.1.2. TREATMENT OF ANIMALS AND CELL CULTURES

Wistar rats of both sexes were used. TGF-β1 (2-10 ng) was injected into the lateral ventricles of 8-day old animals. Control animals received an equal volume of vehicle. Astrocytes were prepared from brains of newborn rats and cultured in Bulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (Spranger et al., 1990, Eur. J. Neurosci. 2:69-76). Contaminating microglia and oligodendrocytes were removed from confluent cultures by shaking and the purity of the cultures was evaluated by staining (Spranger et al., 1990, Eur. J. Neurosci. 2:69-76). Microglial cells were identified by staining with unspecific esterase.

8.1.3. RNA PREPARATION AND NORTHERN BLOTS

RNA was prepared from cultured astrocytes and from various brain regions of adult rats and subjected to quantitative Northern blot analysis (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351; Heumann, R. and Thoenen, H., 1986, J. Biol. Chem. 261:9246-9249). Following electrophoresis and blotting of RNA to nylon filters, they were hybridized as described earlier using ^{32}P-labelled complementary RNA (cRNA) probes. The filters were washed, exposed to x-ray film and the mounts of specific transcripts present (NGF or TGF-β) were determined by laser scanning of the autoradiograms. NGF-cRNA probes were obtained from run-on transcription of the mouse NGF-cDNA clone inserted into the pGem vector (Heumann, R. and Thoenen, H., 1986, J. Biol. Chem. 261:9246-9249). The cDNA for mouse TGF-β1 was a kind gift from Dr. F. Lee, DNAX Inc., California and a SMA1-fragment was subcloned into the Bluescript vector. TGF-β2 riboprobes were obtained using the human TGF-β2-cDNA (deMartin et al., 1987, EMBO J. 60:3673-3677) and a

riboprobe specific for TGF-β3 was constructed from an isolated mouse TGF-β3 genomic clone.

8.1.4. DNA CONSTRUCTS AND TRANSFECTION OF ASTROCYTES

The promoter region of the NGF gene was isolated from a Balb/c mouse genomic library and a 2.1kb Hinc II/PvuII fragment was sequenced, subcloned into the CAT-expression vector pBLCAT3 and used for transfection studies (Hengerer et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3899). For isolation of the promoter region of mouse TGF-β1, the mouse library was screened with two 30-mer oligonucleotides synthesized according to the known sequence for mouse TGF-β1 (Derynck et al., 1986, J. Biol. Chem. 261:4377-4379). A 2kb genomic clone was sequenced and a 1.8kb Xba1/Bgl II fragment was subcloned into the pBLCAT3 vector. A mouse TGF-β3 clone was isolated using the data available for the human TGF-β3 cDNA (Derynck et al., 1988, EMBO J. 7:3737-3743; ten Dijke et al., Proc. Natl. Acad. Sci. U.S.A. 85:4715-4719). Rat astrocytes were transfected overnight with 10ug of the appropriate plasmids as described earlier (Hengerer et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3899). The cells were washed and incubated for 24 hours in DMEM containing 1% (v/v) fetal calf serum in the absence or presence of 2ng TGF-β1. CAT activity was determined in cell extracts using equal amounts of protein (Hengerer et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3899). Nuclei isolated from control and TGF-β-treated astrocytes (6 hours) were incubated as described earlier (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351). The labelled nascent RNA was subsequently hybridized to an excess of cRNA spotted onto nylon filters (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351).

8.2. RESULTS

8.2.1. EFFECT OF TGF-β ON NGF-mRNA IN ASTROCYTES

Astrocyte cultures prepared from rodent brains synthesize and release NGF into the culture medium (Lindsay, R.M., 1979, Nature 282:80-82) and the levels of NGF-mRNA in these cells are influenced by various cytokines and growth factors, such as IL-1 and FGF (Spranger et al., 1990, Eur. J. Neurosci. 2:69-76). However, of all the factors tested on astrocytes, TGF-β1, which is present in serum (Childs et al., 1982, Proc. Natl. Acad. Sci. U.S.A. 79:5312-5316), produced the largest increase in NGF-mRNA. As shown in Figure 15, TGF-β1 markedly (50-fold) elevated NGF-mRNA in astrocytes (lane 2) and this stimulation was virtually abolished by anti-TGF-β-antibodies (Figure 15, lane 3). Actinomycin-D and cycloheximide also blocked the TGF-β1 mediated increase of NGF-mRNA, suggesting that both RNA and protein synthesis are involved in the stimulation (Figure 15, lane 5 and lane 6). Only cycloheximide slightly elevated NGF-mRNA in astrocytes. This stabilizing effect of cycloheximide on NGF-mRNA had also previously been observed with cultured rat fibroblasts (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351). Figure 16a shows that the discernible effect of TGF-β1 on NGF-mRNA was already present with 0.2ng of TGF-β1/ml and that a plateau was reached with about 5ng/ml. Furthermore, NGF-mRNA levels in TGF-β stimulated astrocytes reached a maximum at 24 hours of incubation and declined only slightly thereafter (Figure 16b). TGF-β1 also increased the amount of NGF released into the culture medium. TGF-β2, which is a homologous protein to TGF-β1 (deMartin et al., 1987, EMBO J. 60:3673-3677), increased NGF-mRNA levels in astrocytes to the same extent as TGF-β1.

8.2.2. TGF-β1 INCREASES TRANSCRIPTION OF THE NGF GENE

In order to determine whether TGF-β1 increases transcription of the NGF gene, astrocytes were transfected with a DNA construct containing the NGF promoter linked to a choloramphenicol acetyltransferase (CAT) reporter gene. Figure 17 demonstrates that TGF-β1 increased CAT activity in the transfected cells representing a stimulation of the NGF promoter activity by TGF-β1. These results, showing an enhanced transcription of the NGF gene following TGF-β1 treatments, were further substantiated in nuclear run-on experiments using nuclei from control and TGF-β1 stimulated astrocytes. As shown in Figure 18, TGF-β1 increased the nuclear labelling of transcripts for NGF, c-fos and β-actin, but not that of c-myc.

8.2.3. EXPRESSION OF TGF-β-mRNAS IN GLIAL CELLS

Since TGF-β is known to be synthesized by a great variety of cells (Sporn et al., 1987, J. Cell Biol. 105:1039-1045), we studied whether cultured astrocytes express TGF-β1-mRNA. Figure 19 shows that control rat astrocytes contain low levels of mRNA encoding TGF-β1 (two transcripts are present, 2.5kb and 1.9kb) (Derynck et al., 1986, J. Biol. Chem. 261:4377-4379) and that TGF-β1 itself elevated TGF-β1-mRNA in these cells (Figure 19, lane 3). The time-course of the increase of TGF-β1 mRNA by TGF-β1 in astrocytes was similar to that found for the stimulation of the NGF-mRNA by this factor. Likewise, transfection experiments using a construct containing a region of the mouse TGF-β1 promoter linked to a CAT reporter gene showed that TGF-β1 also stimulated TGF-β1 transcription (Figure 19b). As shown in Figure 20, TGF-β1 also increased rhw mRNA levels of TGF-β3, which is another member of the TGF-β gene family (Derynck et al., 1988, EMBO J. 7:3737-3743; ten Dijke et al., Proc. Natl. Acad. Sci. 85:4715-4719). TGF-β2-mRNA

however, was not detected in either control or in stimulated astrocyte cultures. Moreover, it appears that microglial cells in culture also express TGF-β1-mRNA in relatively high amounts.

8.2.4. EFFECT OF TGF-β1 IN VIVO

To substantiate the results obtained with cultured rat astrocytes, we also studied the effect of injection of TGF-β1 into the rat brain. Figure 21 shows that TGF-β1 injected into the brain ventricles increased the level of NGF-mRNA in rat hippocampus (about 3-fold) albeit to a lower extent than in astrocyte cultures.

8.3. DISCUSSION

The abovementioned results support a role for TGF-β in the regulation of NGF synthesis in the rat CNS. Both TGF-β1 and TGF-β2 produced a 50-fold increase of NGF expression in cultured rat astrocytes and TGF-β1 also increased NGF-mRNA levels in the rat hippocampus in vivo. That the increase in astrocyte NGF-mRNA by TGF-β results from enhanced transcription was shown in transfection studies using an NGF promoter/CAT reporter gene construct and in nuclear run-on studies using nuclei from control and TGF-β-treated cells. Simultaneously with the activation of NGF synthesis, TGF-β1 also increased its own expression in astrocytes and additionally elevated TGF-β3-mRNA. The autocrine stimulation of TGF-β1 expression has recently been reported for cells other than astrocytes (Van Obberghen-Schilling et al., 1988, J. Biol. Chem. 263:7741-7746). The mechanism(s) by which TGF-β enhances NGF transcription is (are) not clear, but TGF-β also increased c-fos expression in astrocytes and we have recently shown that the NGF promoter contains an AP-1 binding site involved in

transcriptional regulation (Hengerer et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:3899).

TGF-β1 was originally identified as a protein inducing anchorage-independent growth of fibroblasts (Roberts et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:5339-5343). Subsequent studies have shown that TGF-β1 exhibits a great variety of biological effects, the protein being particularly abundant in platelets and bone tissue (Sporn et al., 1987, J. Cell Biol. 105:1039-1045; Massague, J., 1987, Cell 49:437-438). However, the occurrence of, and the possible role played by, TGF-β1 in the brain have not been thoroughly studied. Previously, Roberts et al. (Roberts et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:5339-5343) reported on the occurrence of TGF-β1 protein in acid-ether extracts of bovine brain, but the results of recent studies employing in-situ hybridization indicated that TGF-β1 transcripts are not present or are at very low levels in mouse brain (Wilcox, J.N. and Derynck, R., 1988, J. Neurosci. 80:1901-1904). However, we have recently observed, using Northern blot analysis, that both TGF-β1- and TGF-β3-mRNAs are present at low levels in all areas of the rat brain, but the cells expressing these mRNAs have not yet been identified. Interestingly, besides astrocytes, microglial cells (at least in culture) also express TGF-β1-mRNA in relatively high amounts. Macrophages in the periphery have previously been shown to produce TGF-β1, although in an inactive form.

In conclusion, TGF-β1 has been observed to stimulate NGF expression in astrocytes in culture and increase NGF-mRNA levels in the rat hippocampus in vivo. As shown earlier, injections of IL-1 into rat brain also elevated NGF-mRNA (4-fold) in the hippocampus. IL-1 has been shown to increase in brain tissue after lesion (Giulian, D. and Lachman, L.B., 1985, Science 228:497-499),

and we have recently obtained evidence that TGF-β1-mRNA also increases after brain injury. Thus, both cytokines most probably play an important role in the injured brain by increasing the production of neurotrophic factors, which would lead to a protection of damaged responsive neurons.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

REGULATION OF NERVE GROWTH FACTOR SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM

TABLE OF CONTENTS

WHAT IS CLAIMED IS:

1. A method for regulating the levels of nerve growth factor in the central nervous system of a subject comprising administering an effective amount of a cytokine to the subject.

2. The method according to claim 1 in which the level of nerve growth factor is increased.

3. The method according to claim 2 in which the subject has a neurologic disorder.

4. The method according to claim 3 in which the neurologic disorder comprises dementia.

5. The method according to claim 3 in which the neurologic disorder comprises Alzheimer's disease.

6. The method according to claim 3 in which the neurologic disorder comprises damage to the nervous system due to trauma.

7. The method according to claim 3 in which the neurologic disorder comprises damage to the nervous system due to ischemia.

8. The method according to claim 3 in which the neurologic disorder comprises damage to the nervous system due to toxic agents.

9. The method according to claim 3 in which the neurologic disorder comprises damage to the nervous system due to infection.

10. The method according to claim 3 in which the neurologic disorder comprises damage to the nervous system due to malignancy.

11. The method according to claim 3 in which the neurologic disorder comprises a neurodegenerative disorder.

12. The method according to claim 3 in which the neurologic disorder comprises a congenital disorder.

13. The method according to claim 3 in which the neurologic disorder comprises a learning disorder.

14. The method according to claim 2 in which the cytokine is interleukin 1.

15. The method according to claim 2 in which the cytokine is fibroblast growth factor.

16. The method according to claim 2 in which the cytokine is tumor growth factor alpha.

17. The method according to claim 2 in which the cytokine is tumor growth factor beta.

18. The method according to claim 2 in which the cytokine is platelet derived growth factor.

19. The method according to claim 2 in which the cytokine is pidermal growth factor.

20. The method according to claim 2 in which the cytokine is insulin-like growth factor I.

21. The method according to claim 2 in which the cytokine is insulin-like growth factor II.

22. The method of claim 14, 15, 16 or 17 in which the method of administration comprises intracerebroventricular injection.

23. The method according to claim 1 in which the level of nerve growth factor is decreased.

24. The method according to claim 23 in which the subject has a neurologic disorder.

25. The method according to claim 24 in which the neurologic disorder comprises a neurodegenerative disorder.

26. A method for regulating the levels of nerve growth factor in the central nervous system of a subject comprising administering to a subject an effective amount of a substance which alters the levels of a cytokine, which cytokine alters the level of nerve growth factor.

27. The method according to claim 26 in which the level of nerve growth factor is increased.

28. The method according to claim 26 in which the level of nerve growth factor is decreased.

29. The method according to claim 27 or 28 in which the subject has a neurologic disorder.

30. The method according to claim 29 in which the substance is an inhibitor of interleukin-1.

31. The method according to claim 30 in which the substance is a glucocorticoid.

32. The method according to claim 31 in which the substance is dexamethasone.

33. A method of controlling the expression of a protein or peptide of interest comprising exposing a recombinant construct comprising (a) the NGF promoter, or a responsive portion thereof, and (b) a nucleotide sequence encoding the protein or peptide of interest, to a substance which regulates the expression of NGF.

34. The method according to claim 33 in which the protein or peptide of interest is NGF.

35. The method according to claim 33 in which the protein or peptide of interest is BDNF.

36. The method according to claim 33 in which the protein or peptide of interest is CNTF.

37. The method according to claim 33 in which the protein or peptide of interest is neurotrophin-3 (NT-3).

38. The method according to claim 33 in which the protein or peptide of interest is a peptide or protein homologous to at least about six amino acids of NGF.

39. The method according to claim 33 in which the protein or peptide of interest is a peptide or protein homologous to at least about six amino acids of BDNF.

40. The method according to claim 33 in which the protein or peptide of interest is a peptide or protein homologous to at least about six amino acids of NT-3.

41. The method according to claim 33 in which the protein or peptide of interest is a peptide or protein homologous to at least about six amino acids of CNTF.

42. The method according to claim 33 in which the protein or peptide of interest is an enzyme.

43. The method according to claim 33 in which the protein or peptide of interest is choline acetyltransferase.

44. The method of claim 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 in which the substance which regulates the expression of NGF is transforming growth factor beta 1.

45. The method of claim 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 in which the substance which regulates the expression of NGF is interleukin 1.

46. The method of claim 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 in which the substance which regulates the expression of NGF is fibroblast growth factor.

47. The method of claim 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 in which the substance which regulates the expression of NGF is transforming growth factor beta

48. The method of claim 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 in which the substance which regulates the expression of NGF is epidermal growth factor.

49. A recombinant nucleic acid molecule comprising the NGF promoter, or a responsive portion thereof, and a nucleotide sequence encoding a protein or peptide of interest which is not nerve growth factor.

50. The recombinant nucleic acid molecule of claim 49 in which the protein or peptide of interest is brain derived growth factor.

51. The recombinant nucleic acid molecule of claim 49 in which the protein or peptide of interest is ciliary neurotrophic factor.

52. The recombinant nucleic acid molecule of claim 49 in which the protein or peptide of interest is neurotrophin-3.

53. An organism containing the recombinant nucleic acid molecule of claim 49, 50, 51, or 52.

54. The organism of claim 53 which is a bacterium.

55. The organism of claim 53 which is a yeast.

56. The organism of claim 53 which is a eukaryotic cell.

57. The organism of claim 53 which is a non-human transgenic animal.

58. Use of an effective amount of a cytokine, especially of a cytokine according to claims 14 to 21, for regulating the levels of nerve growth factor in the central nervous system of a subject.

FIG.1a

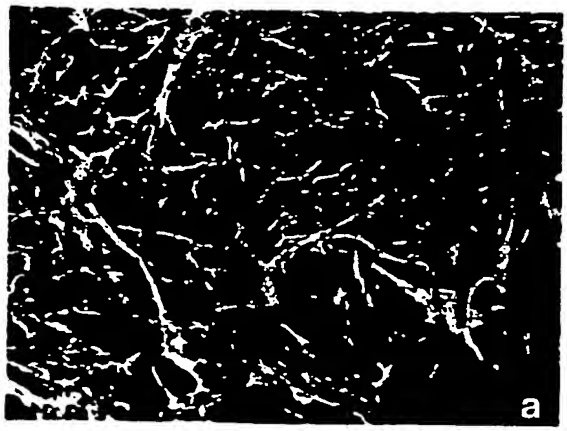


FIG. 1b

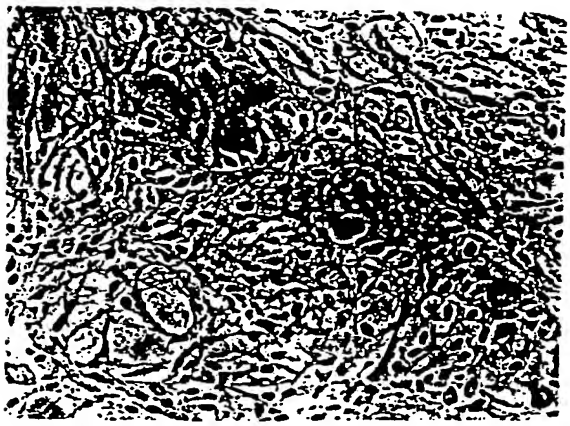


FIG.1c

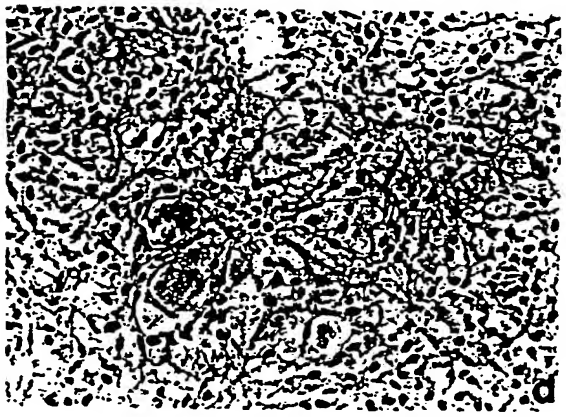


FIG.1d

FIG. 2b

FIG. 2d

FIG. 2a

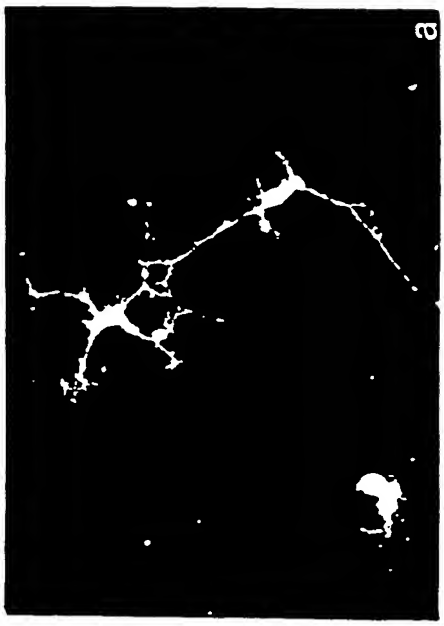
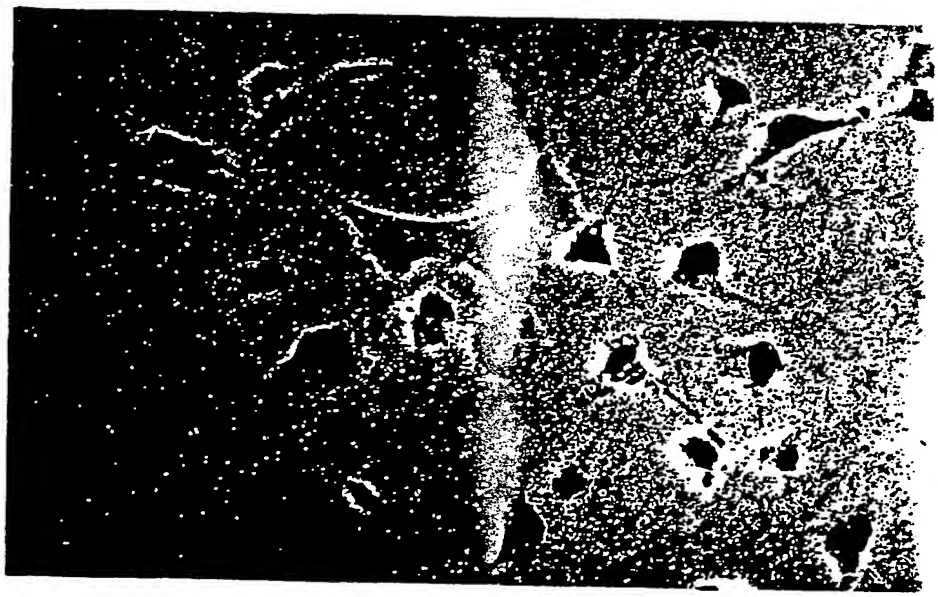


FIG. 2c

FIG. 3a



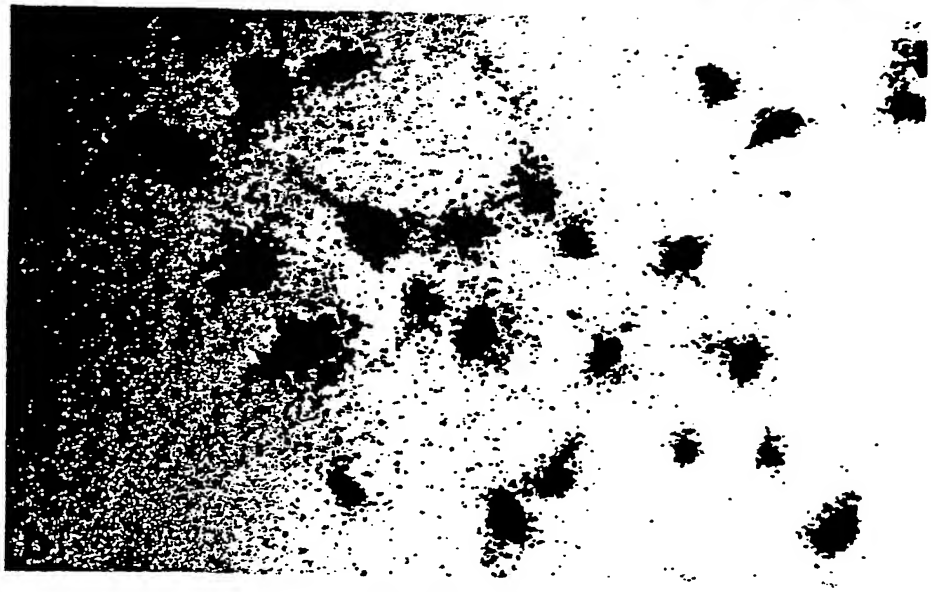


FIG. 3b

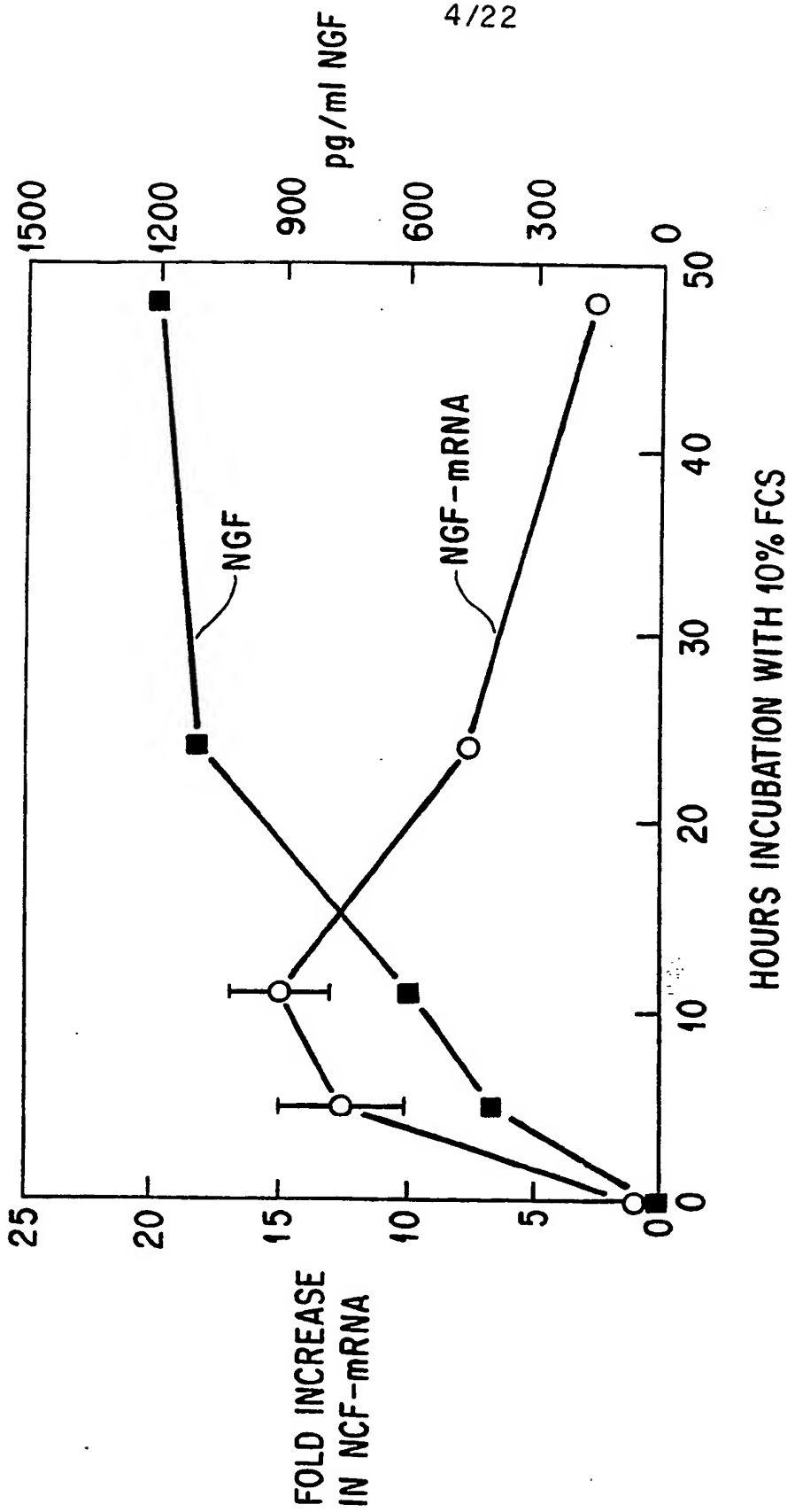


FIG. 4

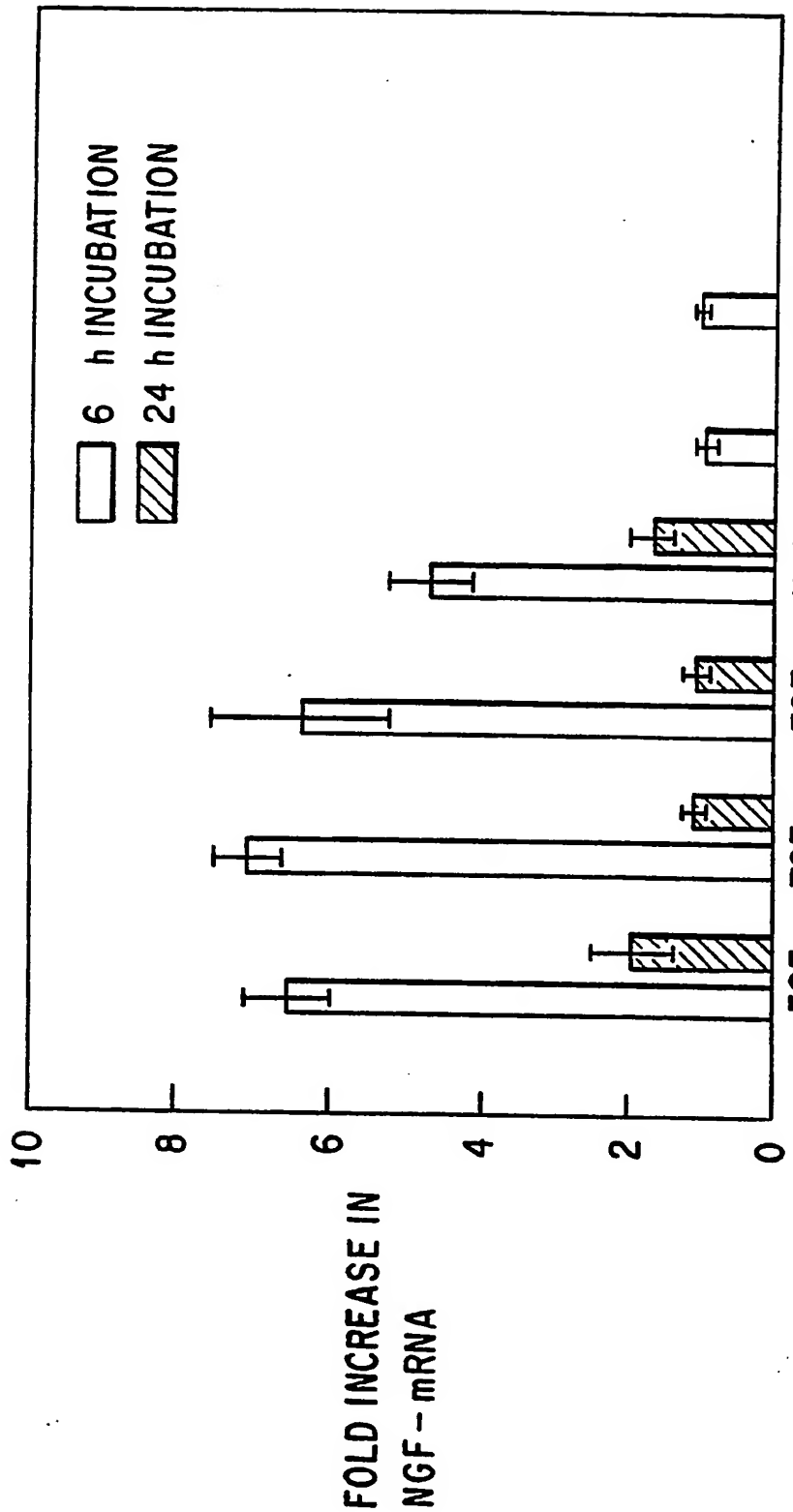


FIG. 5

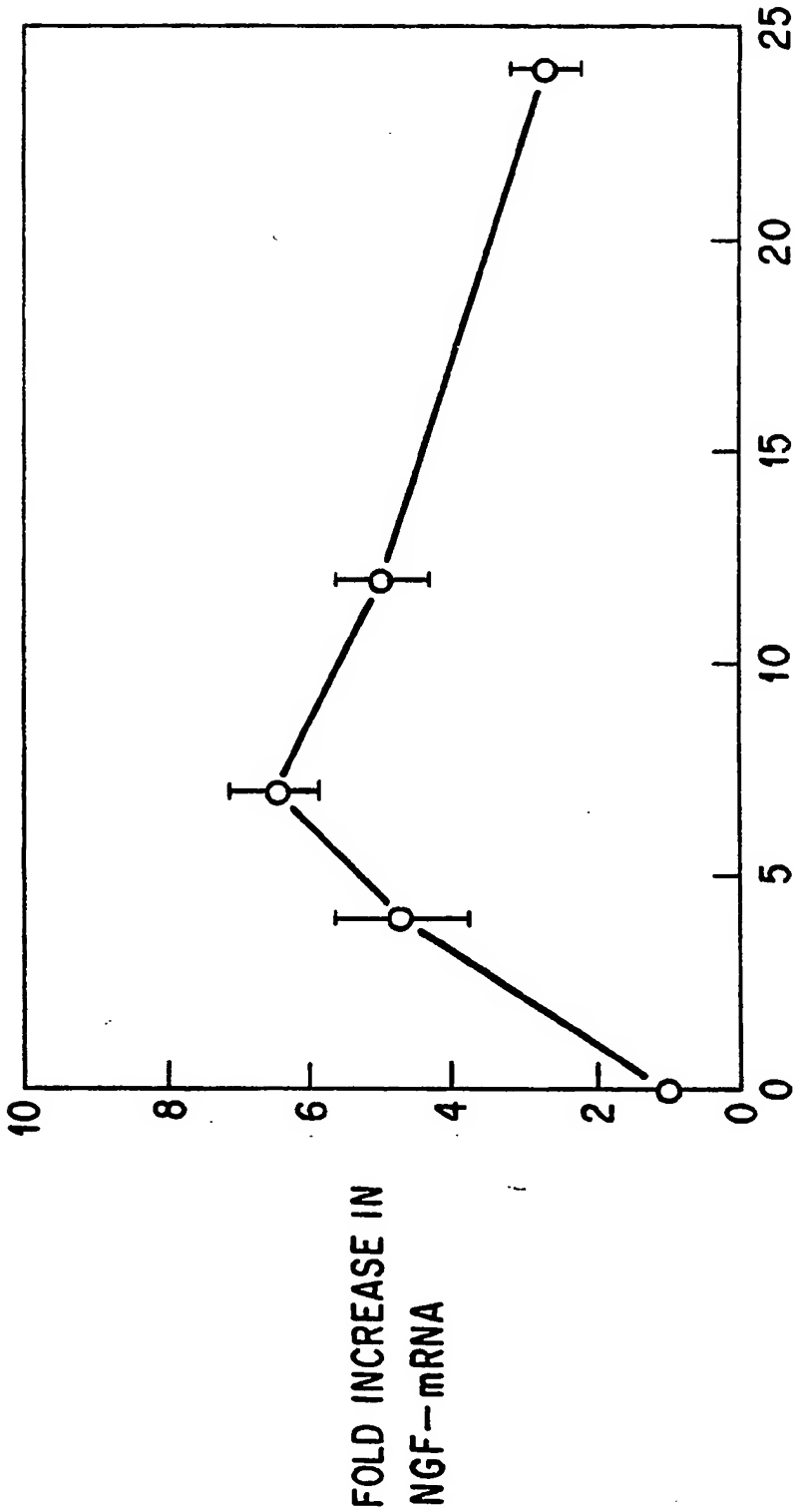


FIG. 6

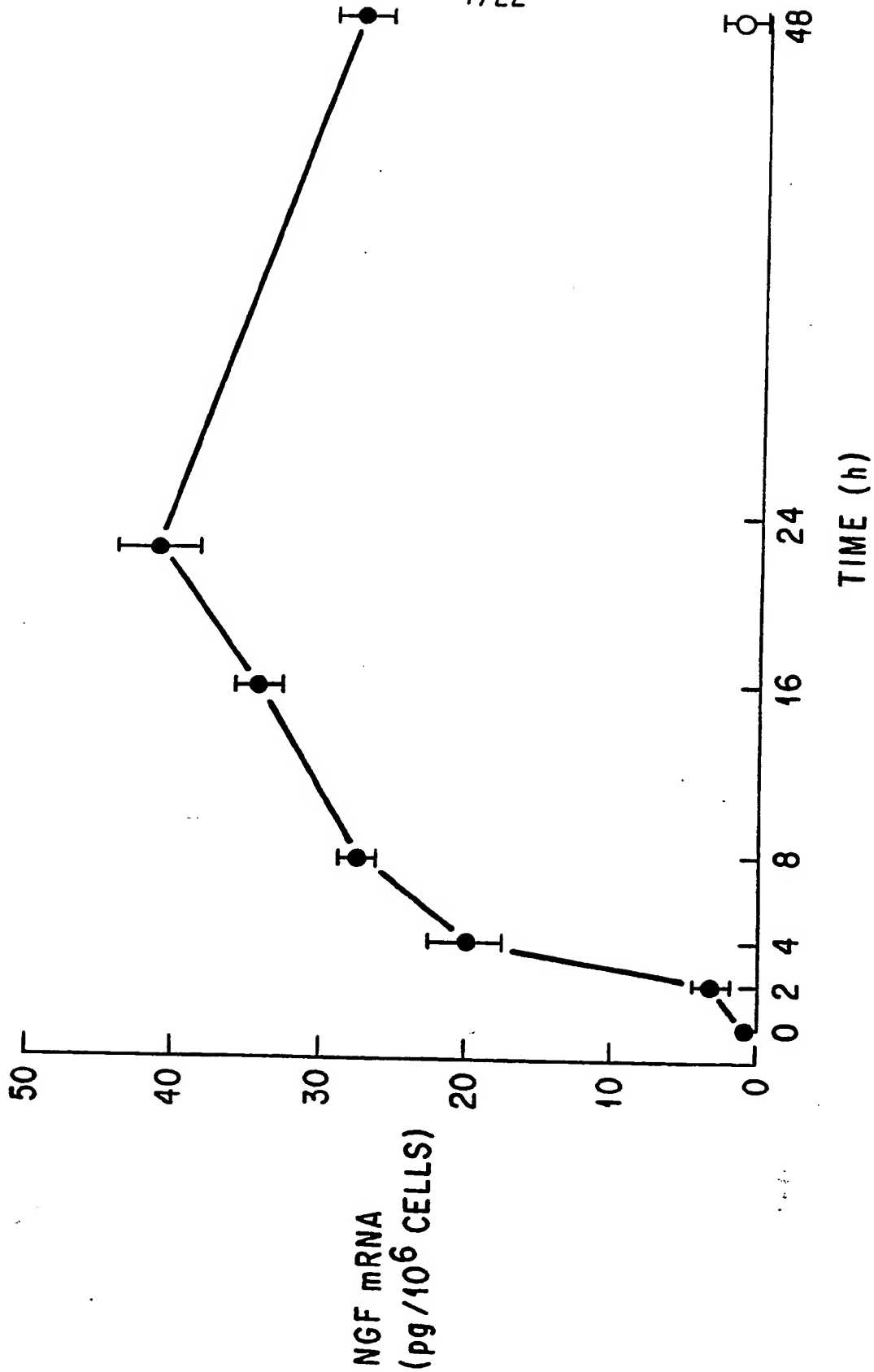


FIG. 7

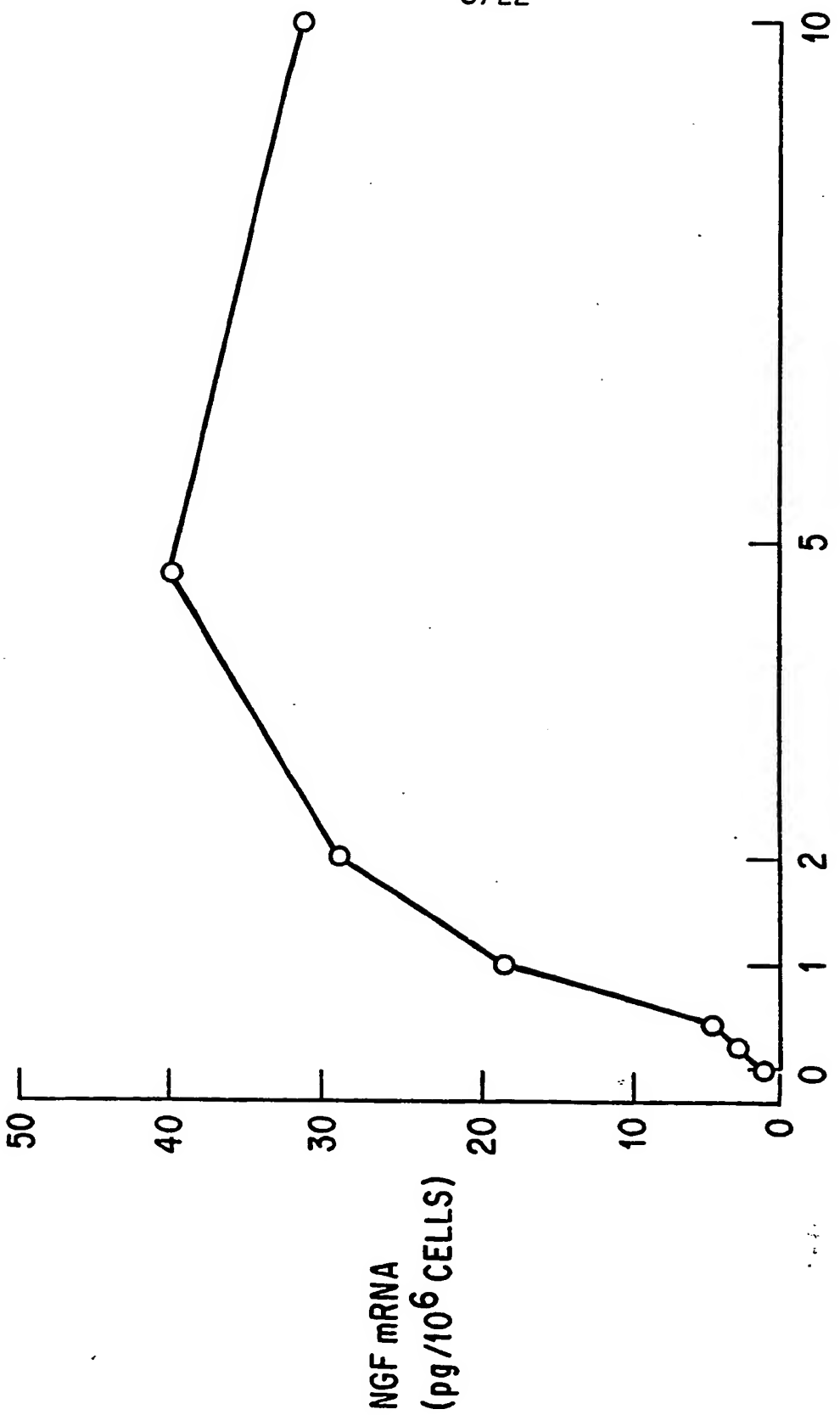


FIG. 8

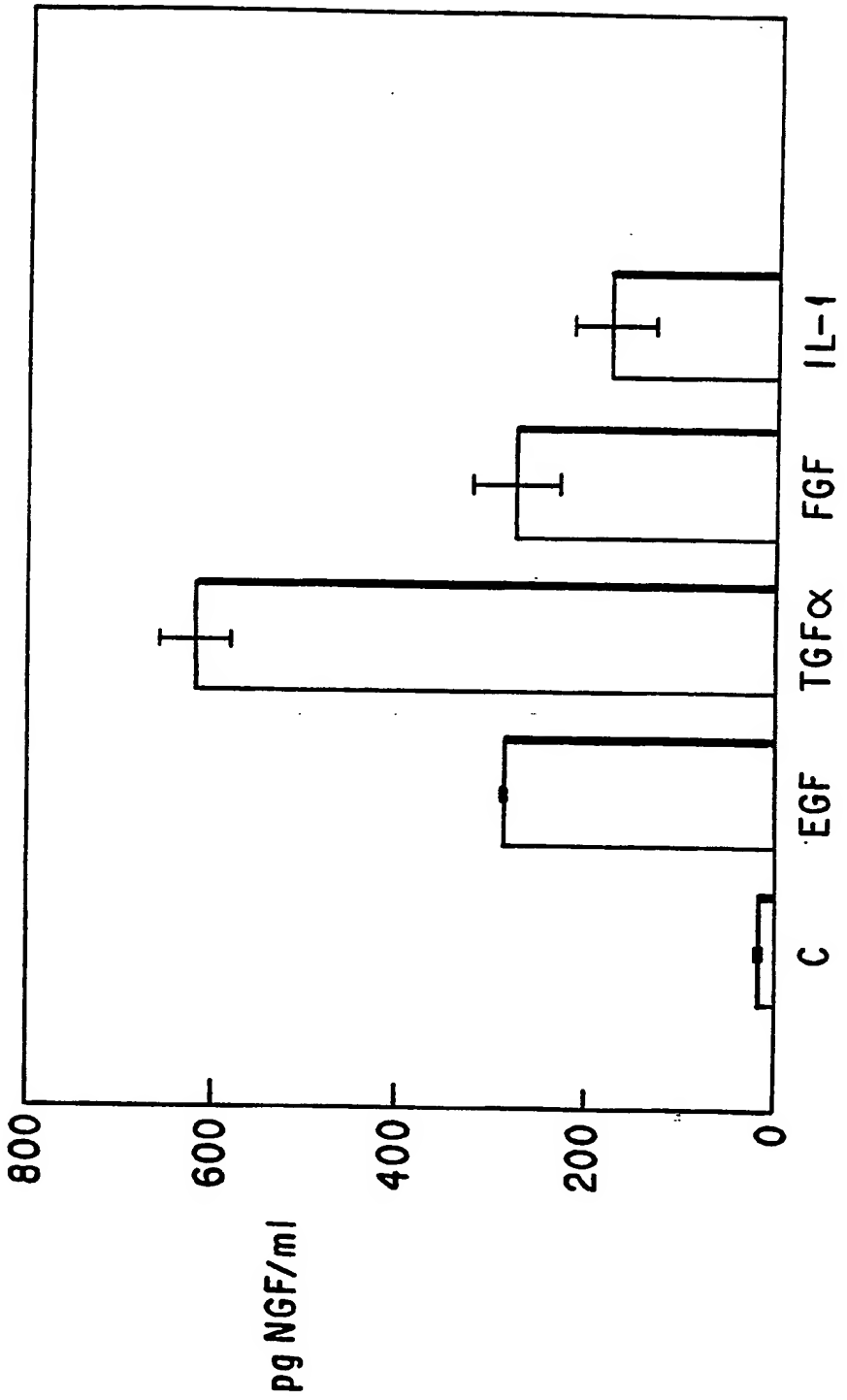


FIG. 9

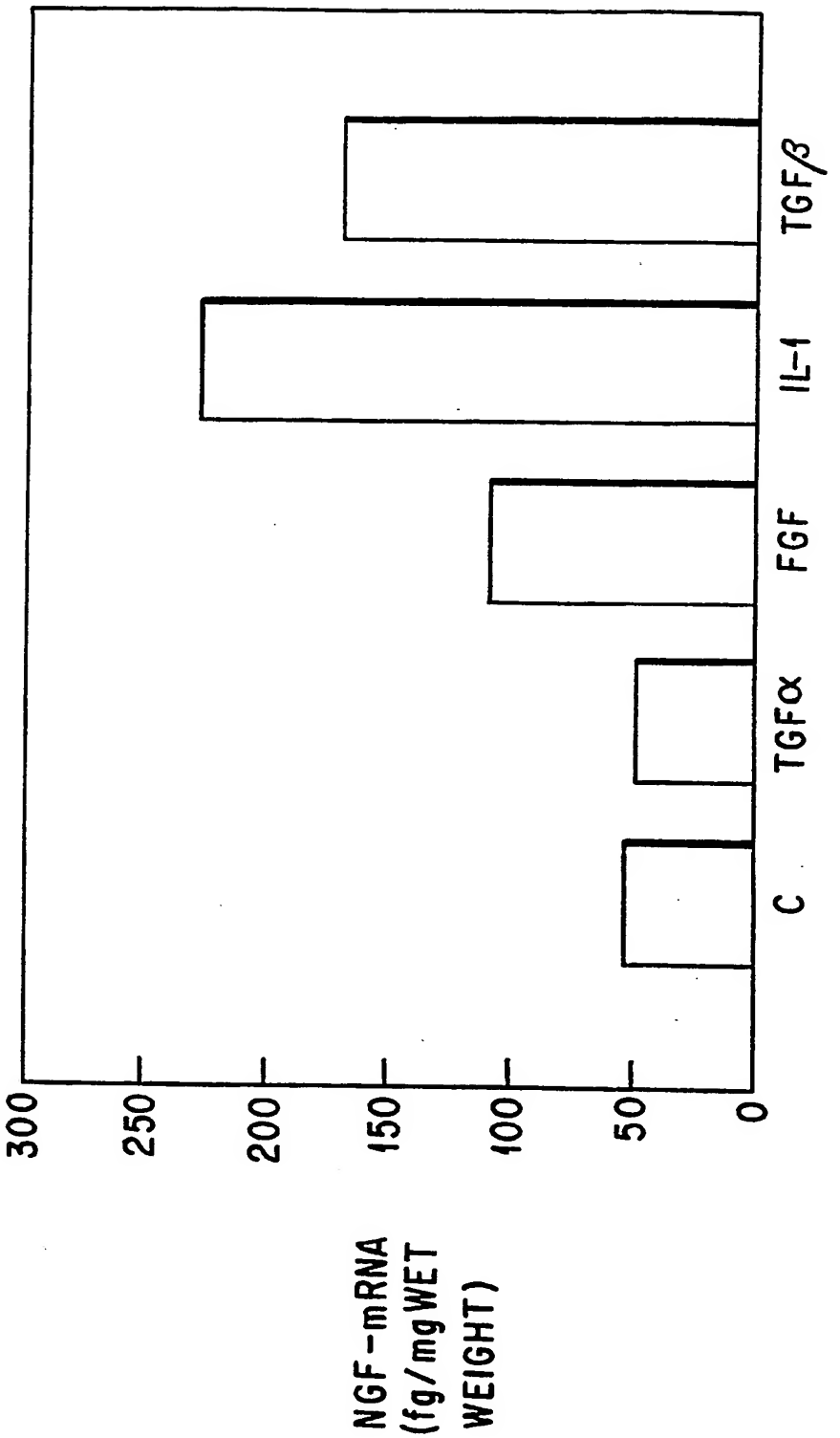


FIG. 10

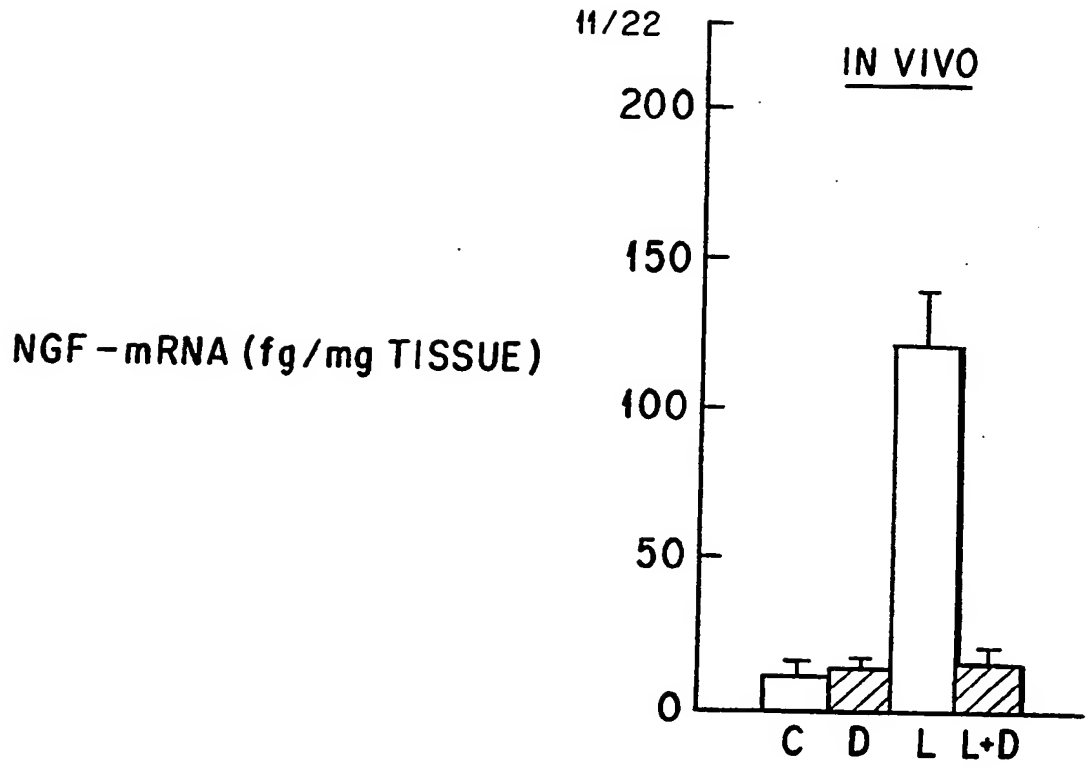


FIG. 11A

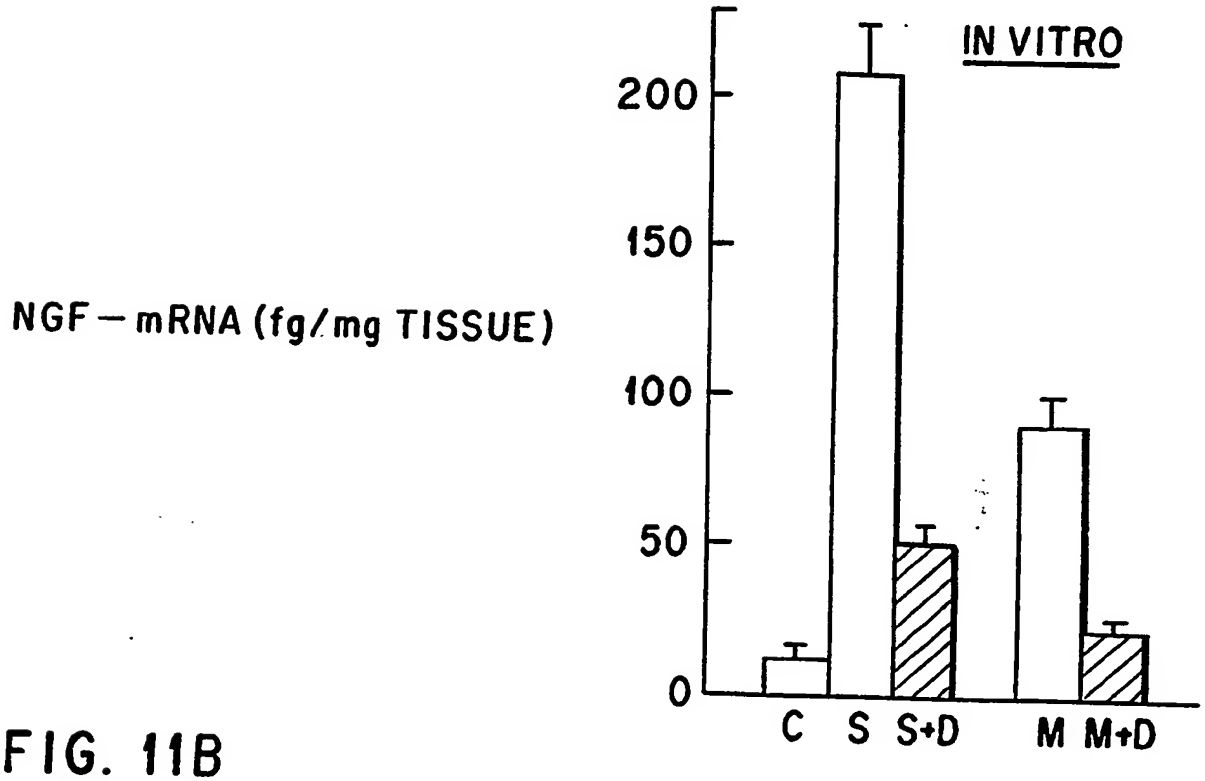


FIG. 11B

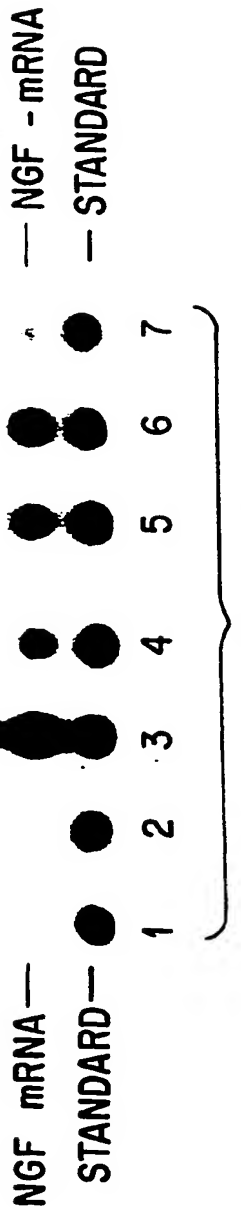


FIG. 12

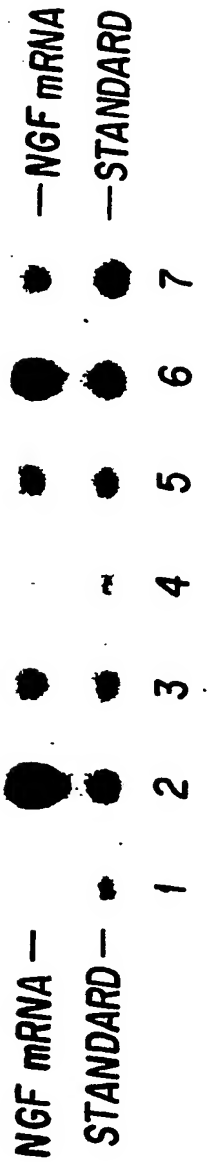


FIG. 13

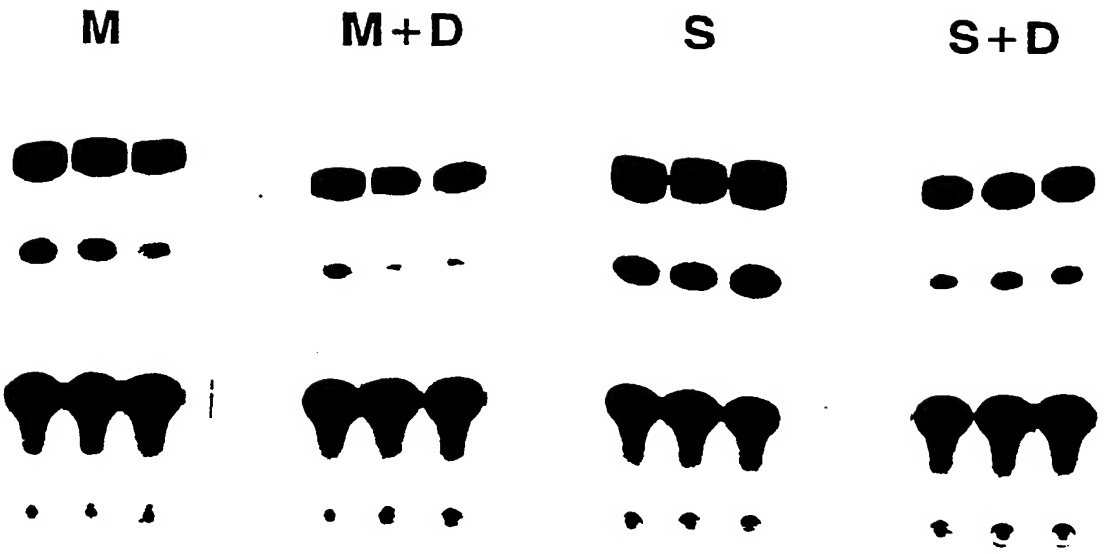


FIG. 14A

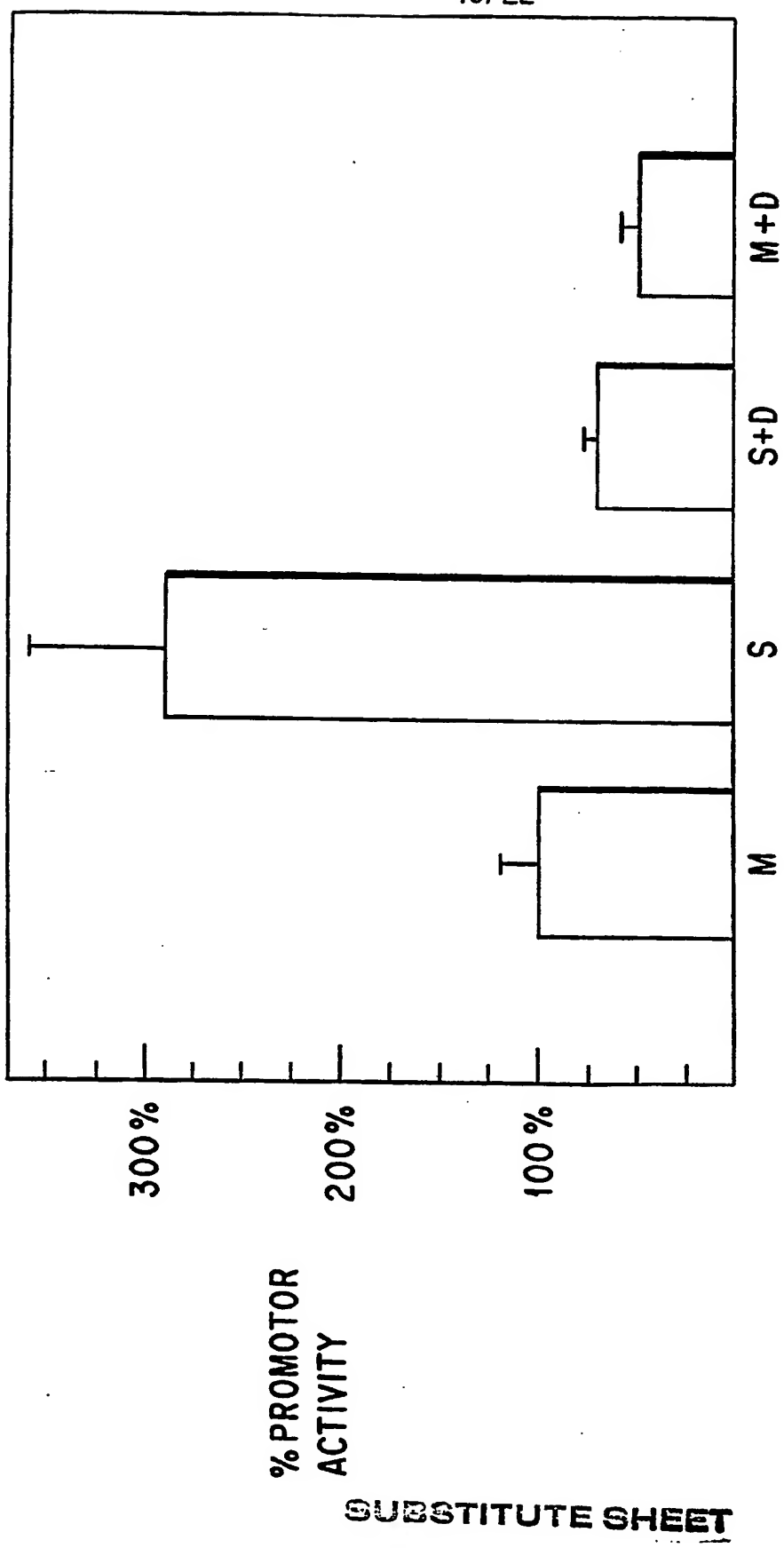


FIG. 14B

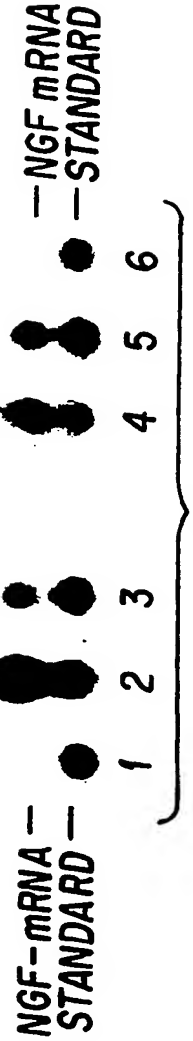


FIG. 15

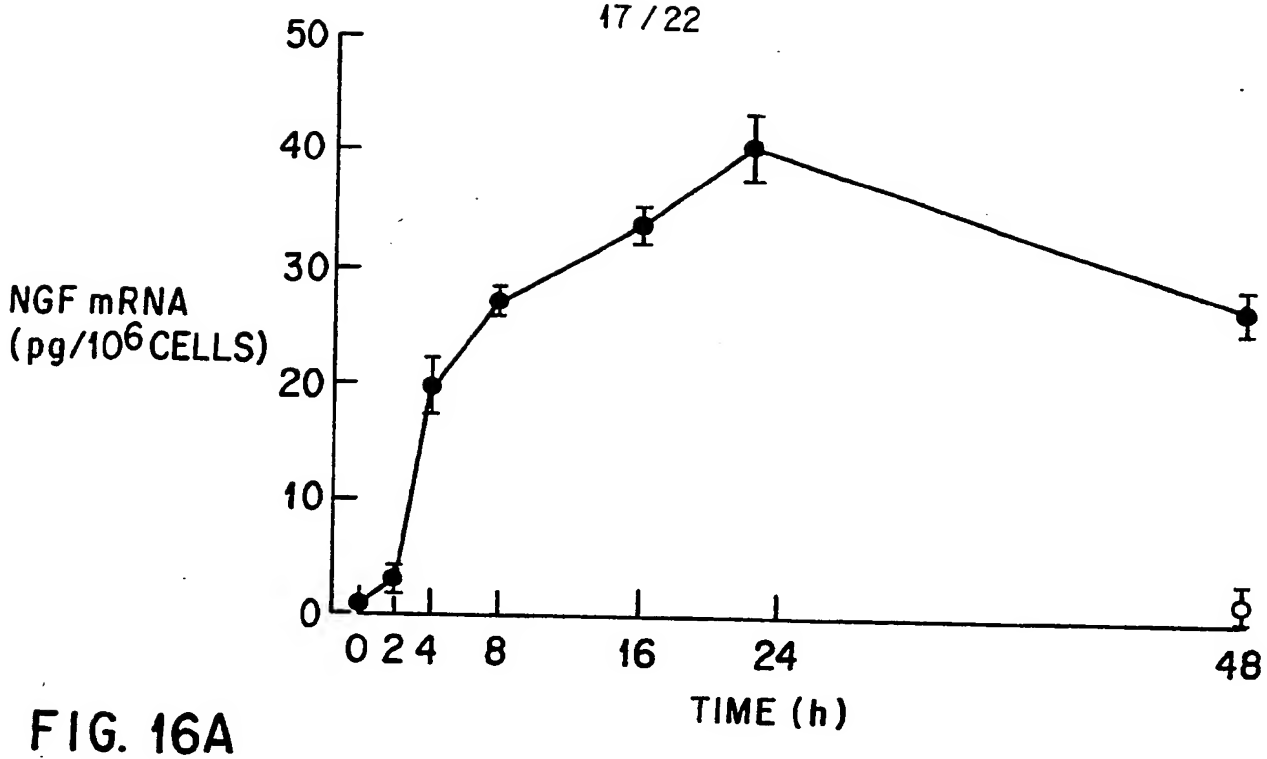


FIG. 16A

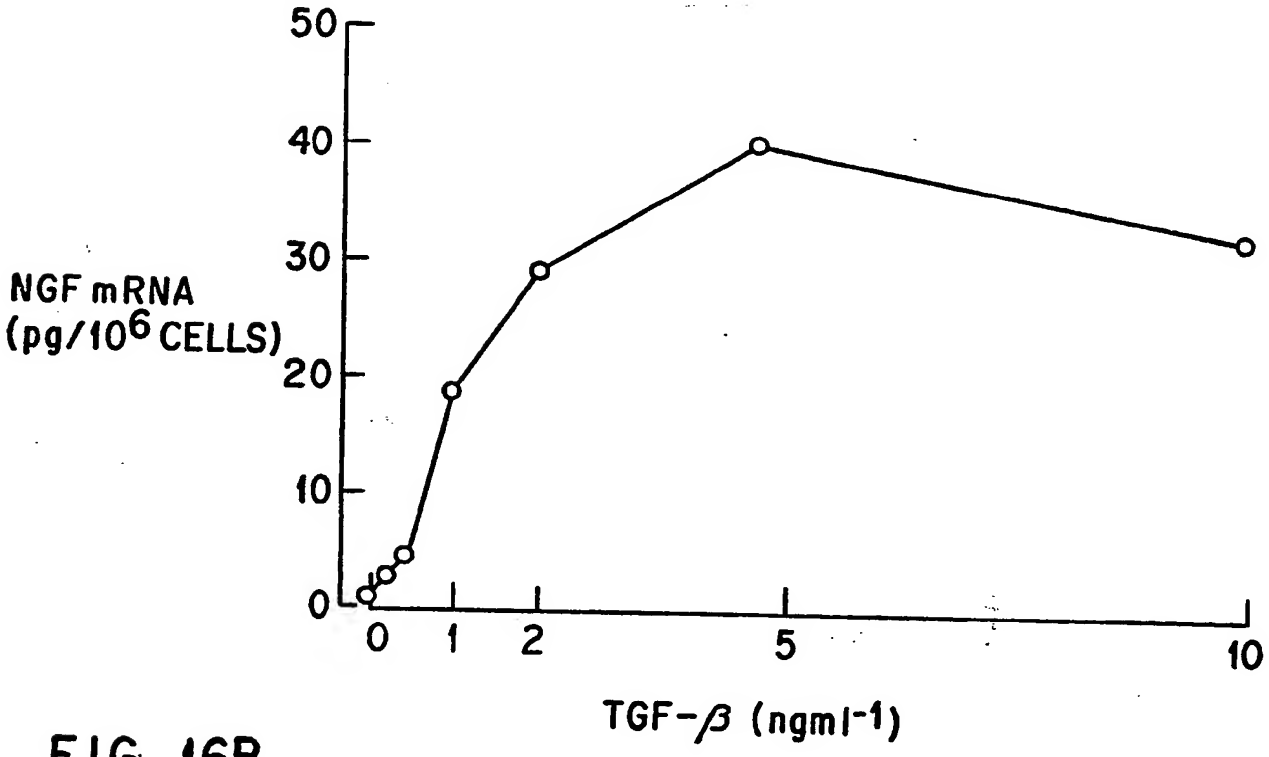


FIG. 16B

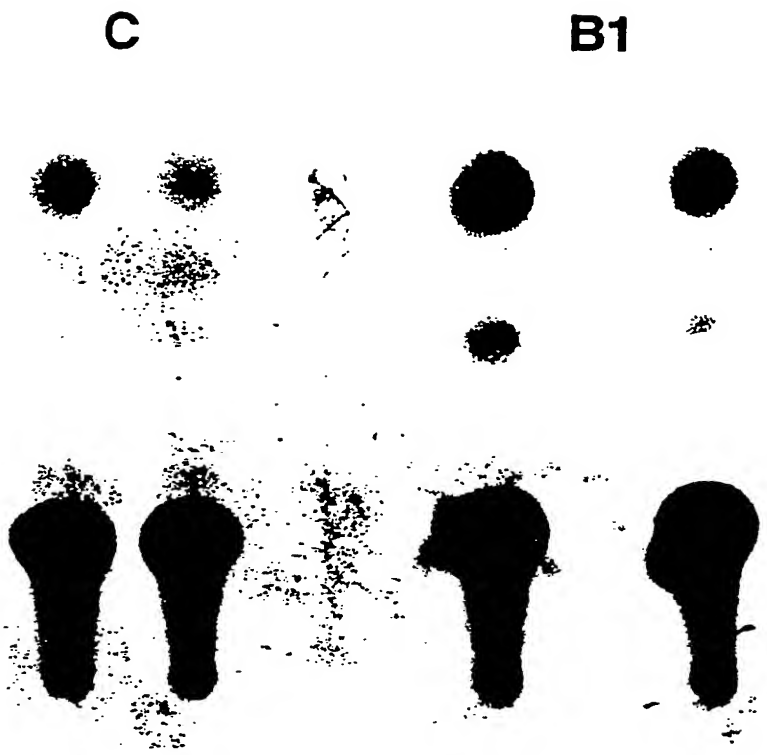


FIG. 17

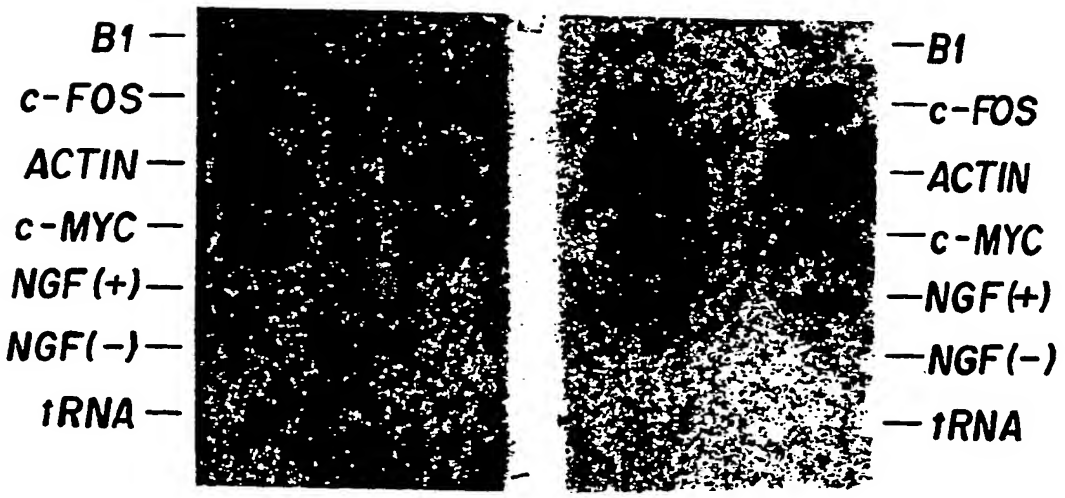


FIG.-18

FIG. 19B

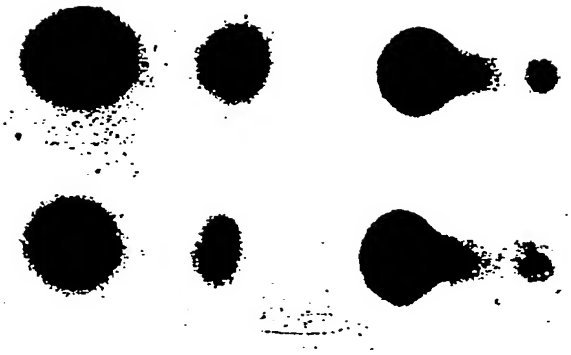


FIG. 19A

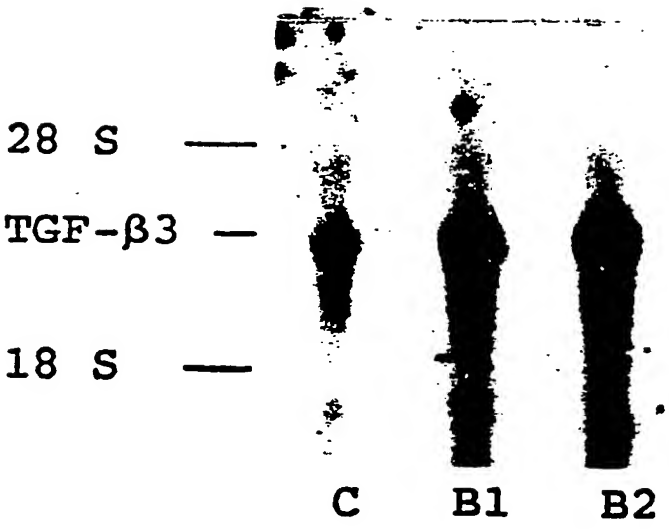


FIG. 20

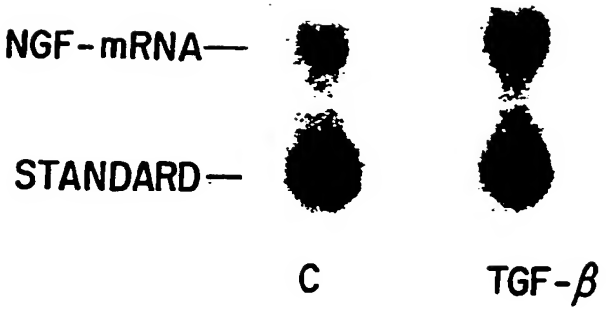


FIG. 21

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01232

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/67, C 12 N 1/21, C 12 N 5/10, C 12 N 15/00, C 12 N 15/16

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Proc. Natl. Acad. Sci. USA, vol. 87, May 1990, B. Hengerer et al.: "Lesion-induced increase in nerve growth factor mRNA is mediated by c-fos", pages 3899-3903 see the whole article cited in the application	33,43,49,53, 56
X	Molecular Brain Research, vol. 3, 1988, Elsevier Science Publishers B.V., M. Zheng et al.: "Structural and functional analysis of the promoter region of the nerve growth factor gene", pages 133-140 see the whole article	49,53,56
Y		33
Y	WO, A, 89/02472 (AMRAD CORP. LTD) 23 March 1989 see the whole document; particularly claims 1-5	33

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th October 1990	28. 11. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION C NTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers *, because they relate to subject matter not required to be searched by this Authority, namely:

*claims 1-32,58
see PCT Rule 39.1(iv)

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9001232 SA 38870

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/11/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8902472	23-03-89	AU-A- 2524888	17-04-89
		EP-A- 0391911	17-10-90